The activation mechanisms of G protein-coupled receptors : the case of the adenosine A2B and HCA2/3 receptors

Liu, R.

Chapter 6

Affinity and kinetics study of anthranilic acids as HCA_2 receptor agonists

This chapter is based upon:

Jacobus P. D. van Veldhoven[#], Rongfang Liu[#], Stephanie A. Thee, Yessica Wouters, Sanne J. M. Verhoork, Christiaan Mooiman, Julien Louvel, Adriaan P. IJzerman

[#] *These authors contributed equally*

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Abstract

Structure-affinity relationship (SAR) and structure-kinetics relationship (SKR) studies were combined to investigate a series of biphenyl anthranilic acid agonists for the HCA_2 receptor. In total, 27 compounds were synthesized and twelve of them showed higher affinity than nicotinic acid. Two compounds, **6g (**IC_{50} **=** 75 nM) and **6z** (IC_{50} = 108 nM) showed a longer residence time profile compared to nicotinic acid, exemplified by their kinetic rate index (KRI) values of 1.31 and 1.23, respectively. The SAR study resulted in the novel 2-F, 4-OH derivative (**6x**) with an IC_{50} value of 23 nM as the highest affinity HCA_2 agonist of the biphenyl series, although it showed a similar residence time as nicotinic acid. The SAR and SKR data suggest that an early compound selection based on binding kinetics is a promising addition to the lead optimization process.

Introduction

The hydroxycarboxylic acid (HCA) receptor family has three members: HCA_1, HCA_2 and HCA_3, which were deorphanized in 2008 (HCA_1, GPR81)[1, 2], 2005 (HCA_2, GPR109A, high affinity nicotinic acid receptor)[3] and 2009 (HCA_3, GPR109B, low affinity nicotinic acid receptor)[4], respectively. They all are G protein-coupled receptors and are predominantly expressed in adipocytes, where they mediate antilipolytic effects through coupling to the $G_{\alpha i}$ protein pathway[5, 6]. Activation of the HCA_2 receptor can have therapeutic benefits, such as an anti-dyslipidemic effect[7], neuroprotective effect[8, 9], and anti-inflammatory effect[10-12]. For the past 50 years nicotinic acid has been used to treat patients who suffer from dyslipidemia, cardiovascular disease and progression of atherosclerosis[13, 14]. However next to this beneficial anti-dyslipidemia effect, nicotinic acid also induces a HCA_2 receptor-mediated side effect of severe flushing, resulting in low patient compliance[15]. Since the cloning and the discovery of the pharmacological role of the HCA_2 receptor activated by nicotinic acid, the interest in the development of novel agonists increased, with many new classes developed in industry[6, 16]. In particular Arena Pharmaceuticals and Merck have been active in this field and their studies have resulted in aminopyrazole-based clinical candidates MK-0354[17, 18] and MK-1903[19], both of which failed in clinical studies due to a lack of efficacy (https://clinicaltrials.gov/ct2/show/NCT00847197). A more recently advanced compound with reportedly little flushing potential is the anthranilic acid derivative MK-6892[20]. Their structures are shown in Figure 1.

In general, the high attrition rates of clinical candidates are often due to a lack of efficacy[21], which led to the realization that equilibrium-derived *in vitro* parameters alone, such as measures of affinity (K_i) or potency (IC_{50}), are not necessarily correlated well with *in vivo* efficacy[22, 23]. A somewhat neglected parameter in early drug discovery, that is, the kinetics (association and dissociation rates, k_{on} and k_{off}) of the interaction between a drug and its target, may be relevant to predict *in vivo* efficacy, witnessed by some recently introduced drugs that favor certain kinetic aspects[23-26]. In particular, the

residence time ($1/k_{off}$) of a drug on its target may be more relevant for its *in vivo* efficacy than the typical *in vitro* equilibrium binding constants, for example, the compound's K_i value. In a survey of 50 drugs on 12 different drug targets, Swinney concluded that long residence time therapeutics often displayed higher efficacy than comparable faster dissociating drugs[27]. For instance, Casarosa et al. found the levels of bronchoprotection *in vivo* by high affinity antagonists of the human muscarinic M3 (hM3) receptor correlated well with their residence time (dissociation half-lives) from the hM3 receptor[28]. Glossop et al. found PF-3635659, a phase II clinical candidate for the treatment of chronic obstructive pulmonary disease (COPD), displayed a very long residence time (slow off-rate binding kinetics) at the M3 receptor mediating a long-lasting bronchodilation *in vivo* of more than one day[29]. However, no kinetics-directed studies on HCA_2 receptor agonists have been published. In this study we aimed to change that while examining the binding kinetics in the early stage of hit to lead optimization of the already extensively investigated class of anthranilic acid derivatives as HCA_2 receptor agonists. As shown in the initial publication by the Merck group, one of their first generation anthranilic acid agonists derived from their original high throughput screening hit is the biphenyl anthranilic acid compound **5a**[30]. Hence, **5a** will be the starting point in this study of both affinity and residence time.

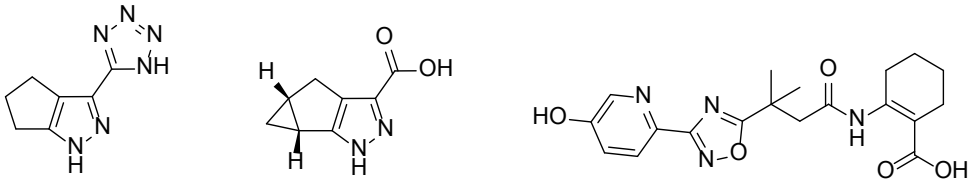


Fig. 1. Structures and potency data of MK-0354[17], MK-1903[19] and MK-6892[20].

Synthesis

The final compounds **5a–f, h–l, n–q** and **6g, m, r–aa** were obtained via two synthetic routes, which are shown in Scheme 1 and Scheme 2. The synthesis in Scheme 1 started from methyl 3-(4-bromophenyl)propanoate[31] (**1**) under Suzuki reaction conditions in dioxane/ethanol as the solvent mixture, the biphenyl compounds **2a–f, i–l, o–p, w** were obtained in good to high yields. Ester **1** was used since with the carboxylic acid analogue[30] as the starting material hard-to-purify mixtures were obtained. Next, saponification of the esters gave the pure carboxylic acids **3a–f, i–l, o–p, w** and under subsequent EDCI*HCl peptide coupling conditions, or via the acid chloride intermediate by the use of $SOCl_2$[30], anthranilic ester amides **4a–f, i–l, o–p, w** and the tetrahydroanthranilic derivative **4z** were isolated, respectively, in low yields. A second saponification of the anthranilic esters and the tetrahydroanthranilic ester, yielded **5a–f, i–l, o–p, w, z** and the successive demethylation of the methoxy compounds **5g, m, w** with BBr_3 in CH_2Cl_2[32] yielded the corresponding hydroxyl derivatives **6g, m, w**.

In Scheme 2 the synthetic approach[30] for compounds **5h, n, q** and **6r–v, x, y, aa** is shown. First, the reaction of 3-(4-iodophenyl)propanoic acid[33] (**7**) and $SOCl_2$ in toluene at 85 °C resulted in the corresponding acid chlorides, and the subsequent nucleophilic substitution with the commercially available methyl 2-aminobenzoate or methyl 2-aminothiophene-3-carboxylate gave both anthranilic ester **8a** in 96% yield and thiophene derivative **8b** in 38% yield. Similar Suzuki conditions as in Scheme 1, except for 6 equivalents instead of 2 equivalents of the base $NaHCO_3$ directly furnished the final Suzuki products as carboxylic acids (**5h, n, q–v, x, y**) after purification in 7–70% yield. The thiophene derivative **5aa** was obtained after the additional saponification of the related ester **4aa** by the use of NaOH (aq.). Finally, similar demethylation conditions as described in Scheme 1 gave the phenolic final compounds **6r–v, x, y, aa** in 2–96% yield as solids.

All final compounds had purities above 95% as determined by HPLC methods and the structures of the compounds were confirmed by ^{1}HNMR spectra.

Results

Both affinity and kinetics

In this study, the non-substituted biphenyl agonist **5a** was used as a starting point in the lead optimization showing an IC_{50} value of 290 ± 104 nM in our hands (Table 1), which is 2.5 times lower in affinity compared to the IC_{50} value of 94 nM reported by Raghavan et al[7]. Next to affinity, compounds with an IC_{50} value < 249 nM (IC_{50} value of nicotinic acid), together with nicotinic acid and **5a**, were tested in a binding kinetics assay using the one-concentration competition association assay with the radioligand [^{3}H]-nicotinic acid. This approach provided the so-called kinetic rate index (KRI) for the compounds, which is mainly driven by the dissociation rate constant (k_{off}) of the ligand–receptor complex[34].

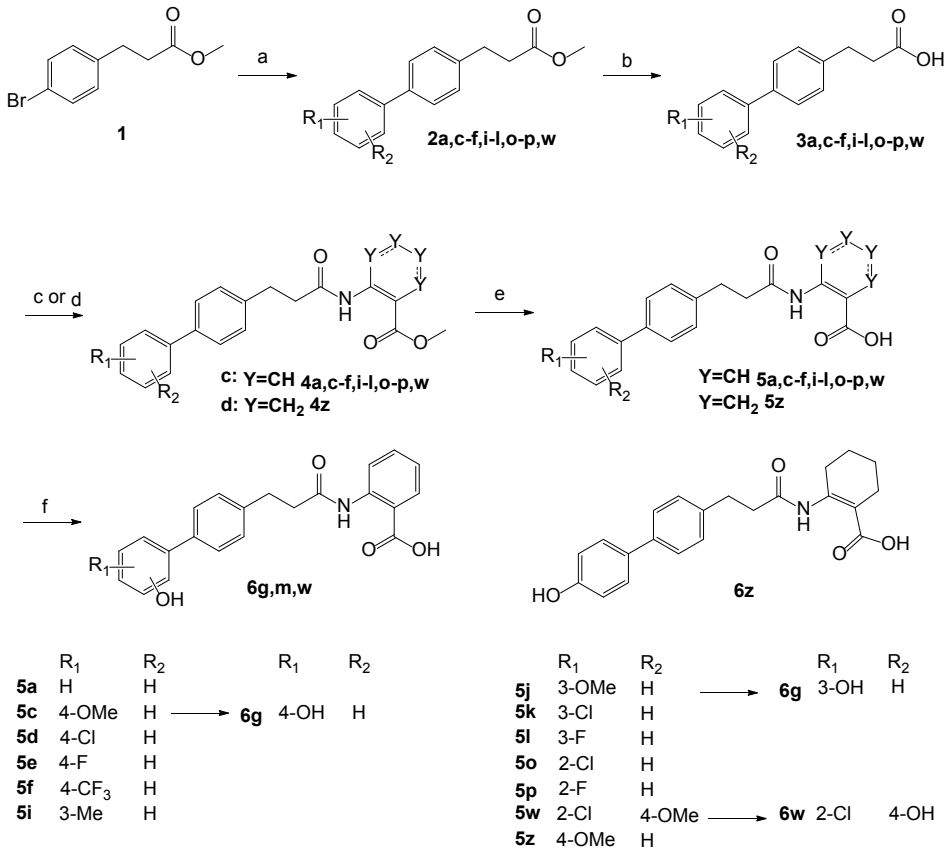
Structure-Affinity Relationships (SAR)

As mentioned before, the non-substituted compound **5a** had an IC_{50} value of 290 ± 104 nM in our hands. Substituents at the 4 position (Table 1) decreased the affinity for the receptor in the cases of 4-Me (**5b**), 4-OMe (**5c**), 4-Cl (**5d**) and 4-CF_3(**5f**). The smaller 4-F (**5e**) was as well accepted (IC_{50} = 323 ± 104 nM) as the non-substituted **5a** by the receptor, but also this compound was still less active than nicotinic acid (Table 1). The hydrogen bond accepting and donating substituent 4-OH (**6g**) resulted in a 4 times improved IC_{50} value of 75 ± 5 nM (Table 1 and Fig. 2), similar to the approx. 6 times enhancement found by the Merck research team[35]. Introduction of a carbon between the aromatic system and the 4-hydroxy group yielding 4-CH_2OH (**5h**) was not allowed given the modest 51% displacement at a concentration of 10^{-5} M (Table 1). Substitutions at the 3-position (**5i–k**) were slightly better accommodated in the binding pocket of the HCA_2 receptor compared to the 4-substituted ones, except for the 3-OH group (**5m**) which showed a 12.5 times reduced affinity compared to the 4-OH substituent (**6g**). Again the smaller 3-F (**5l**) had an affinity (IC_{50} = 283 ± 25 nM) comparable to the non-substituted phenyl compound **5a** (Table 1). The 2-position seems to be preferred for a broader range of substituents (Table 1). Lipophilic groups such as 2-Me (**5n**) and 2-CF_3 (**5q**) were tolerated better at the 2-position

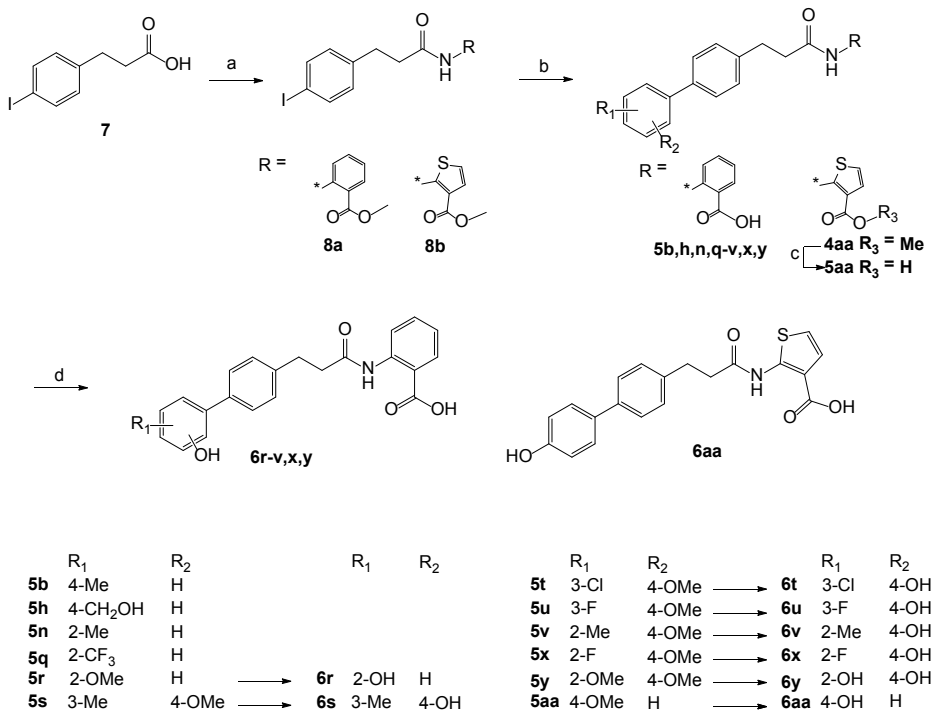
in contrast to the other positions, but both gave decreased affinities with respect to the non-substituted **5a**. Both halogens 2-Cl (**5o**) and 2-F (**5p**) increased the affinity to 94 ± 6 nM (19 nM in Ding et al.[36]) and 83 ± 18 nM, respectively. Also the polar 2-OH (**6r**) resulted in an improved IC_{50} value of 86 ± 13 nM.

Both of the abovementioned affinities of the mono-substituted analogues at the 2-position and, to a lesser extent, the 3-position indicate there is space in the binding pocket. Thus in combination with the highly preferred 4-OH substituent all of the di-substituted 3-R, 4-OH (**6s–u**) and 2-R, 4-OH (**6v–y**) compounds gained affinity when compared to their mono-substituted analogues (**5i, k, l** and **5n–p, 6r**) (Table 2). The novel 3,4-disubstituted compounds 3-Me, 4-OH (**6s**), 3-Cl, 4-OH (**6t**), and 3-F, 4-OH (**6u**) showed IC_{50} values of 201 ± 34, 365 ± 96 and 91 ± 20 nM, respectively, which resulted in a 3–4 times increased affinity with respect to **5i, k, l**. The 2,4-disubstituded **6v–y** were better tolerated in the receptor compared to the 3,4-disubstituted congeners (**6s–u**), as was also seen in the mono-substituted compounds. All of the 2-R, 4-OH compounds had higher affinities than the threshold value of IC_{50} = 249 nM (IC_{50} value of nicotinic acid) and were novel, except for **6w** with the often used substituent pattern 2-Cl, 4-OH[7, 35]. Again the 2-Me, 4-OH (**6v**); 2-Cl, 4-OH (**6w**) and 2-F, 4-OH (**6x**) displayed approx. 3–4 times improved IC_{50} values of 129 ± 19, 34 ± 2 and 23 ± 3 nM over the mono-substituted compounds (**5n–p**). The 2,4-diOH derivative (**6y**) resulted in an 1.4 times increase in affinity over **6r**. Novel tetrahydroanthranilic acid derivative (**6z**) (Fig. 2) and the thiophene bioisostere (**6aa**) of anthranilic acid compound **6g** (4-OH) resulted in slightly decreased affinities but well below the threshold (Table 3).

In summary, the novel 2-F, 4-OH (23 ± 3 nM) derivative showed the highest affinity in this series. In order to address the selectivity of the novel compounds a functional yeast growth assay was performed showing that the compounds are only active on the HCA_2, not HCA_3 receptor (Fig. S1).



Scheme 1. Reagents and conditions: (a) appropriate phenylboronic acid, $Pd(PPh_3)_4$, 1 M $NaHCO_3$ (aq), dioxane, ethanol, 100 °C, 3 h, microwave; (b) 5 M NaOH (aq.), dioxane, room temperature, 3 h; (c) methyl-2-amino-benzoate, EDCI*HCl, DMAP, CH_2Cl_2, room temperature, 72 h; (d) i) **3c**, $SOCl_2$, reflux, 1.5 h ii) methyl 2-amino-1-cyclohexene-1-carboxylate, toluene at 70 °C overnight; (e) 5 M NaOH (aq.), dioxane, room temperature, 24 h; (f) 1M BBr_3, CH_2Cl_2, -78 °C to room temperature, 3 h.

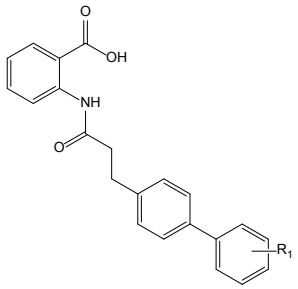


	R_1	R_2			R_1	R_2
5b	4-Me	H				
5h	4-CH_2OH	H				
5n	2-Me	H				
5q	2-CF_3	H				
5r	2-OMe	H	→	**6r**	2-OH	H
5s	3-Me	4-OMe	→	**6s**	3-Me	4-OH

	R_1	R_2			R_1	R_2
5t	3-Cl	4-OMe	→	**6t**	3-Cl	4-OH
5u	3-F	4-OMe	→	**6u**	3-F	4-OH
5v	2-Me	4-OMe	→	**6v**	2-Me	4-OH
5x	2-F	4-OMe	→	**6x**	2-F	4-OH
5y	2-OMe	4-OMe	→	**6y**	2-OH	4-OH
5aa	4-OMe	H	→	**6aa**	4-OH	H

Scheme 2. Reagents and conditions: (a) i) $SOCl_2$, reflux, 18 h under N2 atmosphere ii) methyl 2-aminobenzoate, toluene, room temperature, 18 h; (b) substituted phenyl boronic acid, PPh_3, $Pd(OAc)_2$, 1 M $NaHCO_3$ (aq.), dioxane, ethanol, 100 °C, 3 h, microwave; (c) 5 M NaOH (aq.), dioxane, room temperature, 24 h; (d) BBr_3, CH_2Cl_2, -78 °C to room temperature, 3 h.

Table 1 Structure-affinity relationships and Structure-kinetics relationships of 2-(monosubstituted biphenyl-3-propanamido)benzoic acids of **5a-f, h-l, n-q** and **6g, m, r.**



Compound	R_1	IC_{50} (nM) or % displacement[a]	KRI[b]
[^{3}H]nicotinic acid			0.70 ± 0.02
nicotinic acid		249 ± 48	0.80 ± 0.07
5a	H	290 ± 104	0.78 ± 0.04
5b	4-Me	68% (65, 72)	—
5c	4-OMe	46% (45, 48)	—
5d	4-Cl	60% (59, 61)	—
5e	4-F	323 ± 41	—
5f	4-CF_3	41% (43, 40)	—
6g	4-OH	75 ± 5	1.31 ± 0.06**
5h	4-CH_2OH	51% (58, 44)	—
5i	3-Me	800 ± 54	—
5j	3-OMe	769 ± 58	—
5k	3-Cl	1401 ± 245	—
5l	3-F	283 ± 25	—
6m	3-OH	940 ± 79	—
5n	2-Me	555 ± 164	—
5o	2-Cl	94 ± 6	0.79 ± 0.02
5p	2-F	83 ± 18	0.92 ± 0.10
5q	2-CF3	326 ± 78	—
6r	2-OH	86 ± 13	0.71 ± 0.03

[a] Percentage displacement of **5b-d**, **5f** and **5h** from single point [^{3}H]nicotinic acid displacement binding assay at 10^{-5} M of cold ligand (N = 2, individual values in parentheses). IC_{50} values in nM ± SEM (n = 3) from full curves of [^{3}H]nicotinic acid displacement binding assay.

[b] Averaged Kinetic Rate Index (KRI, individual values in parentheses), determined in the absence or presence (1 × IC_{50} value) of nicotinic acid, **5a**, **6g**, **5o**, **5p**, or **6r**, as the ratio of specific binding at time points t_1 = 7 minutes and t_2 = 90 minutes. Statistical evaluation (*p*-values) of KRI values was performed by a two-tailed homoscedastic Student's *t*-test using non-radioactive nicotinic acid as reference ligand; significance is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

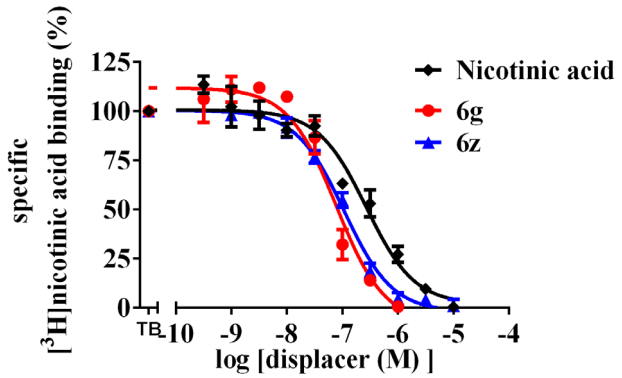
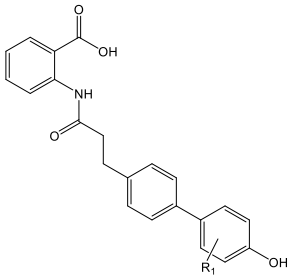


Fig. 2. Displacement of specific [^{3}H]nicotinic acid binding from the human HCA_2 receptor by nicotinic acid, **6g** and **6z** to reveal their affinity values. The assay was performed on HEK293T-$hHCA_2$ membranes. The mean curves of three independent experiments performed in duplicate are shown.

Table 2. Structure-affinity relationships and Structure-kinetics relationships of 2-(4-hydroxy-2 or 3 disubstituted biphenyl-3-propanamido)benzoic acids **6s-y.**



compound	R_1	IC_{50} (nM)[a]	KRI[b]
6s	3-Me	201 ± 34	0.83 ± 0.07
6t	3-Cl	365 ± 96	—
6u	3-F	91 ± 20	0.89 ± 0.06
6v	2-Me	129 ± 19	0.90 ± 0.04
6w	2-Cl	34 ± 2	0.98 ± 0.04
6x	2-F	23 ± 3	0.91 ± 0.12
6y	2-OH	58 ± 12	0.92 ± 0.09

[a] IC_{50} values in nM ± SEM (n = 3) from full curves of [^{3}H]nicotinic acid displacement binding assay.

[b] Averaged Kinetic Rate Index (KRI, individual values in parentheses), determined in the presence (1 × IC_{50} value) of **6s** or **6u-6y**, as the ratio of specific binding at time points t_1 = 7 minutes and t_2 = 90 minutes. Statistical evaluation (*p*-values) of KRI values was performed by a two-tailed homoscedastic Student's *t*-test using non-radioactive nicotinic acid as reference ligand; significance is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Structure-Kinetics Relationships (SKR)

Kinetics of [^{3}H]-nicotinic acid

The association and dissociation experiments of [^{3}H]nicotinic acid (15 nM) were conducted at 25 °C on HEK293T-hHCA$_2$ membranes (35 μg of protein per well). The observed association rate constant, k_{obs} = 0.16 ± 0.03 min^{-1}, was calculated from the association binding experiment. The dissociation rate constant, k_{off}, was 0.27 ± 0.04 min^{-1}. Representative association and dissociation graphs are shown in Figure 3A and B, respectively.

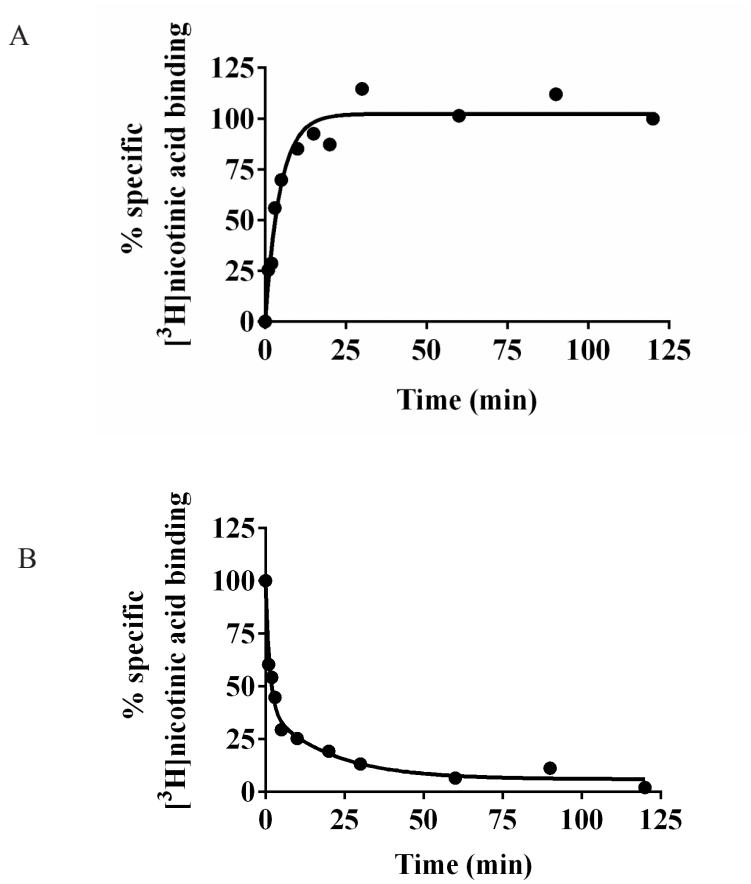


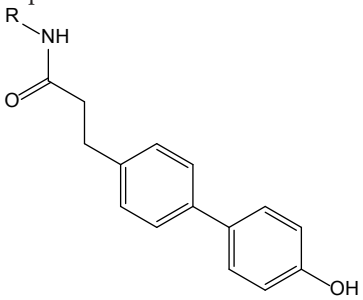
Fig. 3. The association (A) and dissociation (B) kinetics of [^{3}H]nicotinic acid binding at the hHCA$_2$ receptor at 25 °C. The assay was performed on HEK293T-hHCA$_2$ membranes. Representative graphs from one experiment performed in duplicate at same conditions.

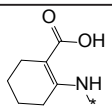
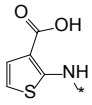
Qualitative kinetics of anthranilic acid derivatives

Besides nicotinic acid, 12 compounds (**5a**, **5o**, **p** and **6g**, **r**, **s**, **u–y**, **z**, **aa**) had affinities < 249 nM (IC_{50} value of nicotinic acid) and these compounds were tested in a one-concentration competition association assay with the radioligand [^{3}H]nicotinic acid (Fig. S2). This provided the data for the determination of a kinetic rate index (KRI) value, which was obtained by dividing the specific radioligand binding measured at t_1 = 7 min (B_{t1}) by the binding at t_2 = 90 min (B_{t2}) in the presence of unlabeled competing ligands (KRI = B_{t1}/B_{t2}). With only the radioligand [^{3}H]nicotinic acid present a KRI value of 0.7 was obtained in the competition association assay (Fig. 4). In the competition association experiments with the reference agonist nicotinic acid or the non-substituted **5a**, the corresponding KRI values were both 0.8, very similar to the KRI value and kinetics of the radioligand. Similar patterns were seen with the 2-Cl (**5o**, KRI = 0.8), 2-F (**5p**, KRI = 0.9) and 2-OH (**6r**, KRI = 0.7) substituents. In contrast, the 4-OH derivative (**6g**) showed a significantly increased KRI value of 1.31 ± 0.06 ($p < 0.005$), which together with the typical profile of the curve in Figure 4 indicates a relatively long residence time on the receptor equivalent to a slow k_{off}. This made **6g** a logical starting point for a series of disubstituted compounds (Table 2). However, 3-substituted, 4-OH derivatives **6s** (3-Me, 4-OH) and **6u** (3-F, 4-OH) had decreased KRI values of 0.83 and 0.89, respectively. This trend was also observed for all the 2-R, 4-OH compounds **6v–y**, including the known 2-Cl, 4-OH derivative (**6w**, KRI = 0.98).

The influence of the anthranilic acid moiety itself was investigated by replacing it for a tetrahydroanthranilic moiety (**6z**) resulting in a similar higher KRI value of 1.23 ± 0.03 ($p < 0.005$) (Table 3) as the related anthranilic acid derivative with the 4-OH substituent (**6g**). However, replacement by the aromatic thiophene bioisostere (**6aa**) yielded a lower KRI value of 0.95 again (Table 3), which indicates that the binding kinetics can also be influenced by this part of the ligand.

Table 3. Structure-affinity relationships and Structure-kinetics relationships of tetrahydroanthranilic acid analogue and thiophene-anthranilic acid bioisostere **6z** and **6aa**.



Compound	R	IC_{50} (nM)[a]	KRI[b]
6z		108 ± 4	1.23 ± 0.03 **
6aa		105 ± 12	0.95 ± 0.02

[a] IC_{50} values in nM ± SEM (n = 3) from full curves of [^{3}H]nicotinic acid displacement binding assay.
[b] Averaged Kinetic Rate Index (KRI, individual values in parentheses), determined in the presence (1 × IC_{50} value) of **6z** and **6aa**, as the ratio of specific binding at time points t_1 = 7 minutes and t_2 = 90 minutes. Statistical evaluation (*p*-values) of KRI values was performed by a two-tailed homoscedastic Student's *t*-test using non-radioactive nicotinic acid as reference ligand; significance is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

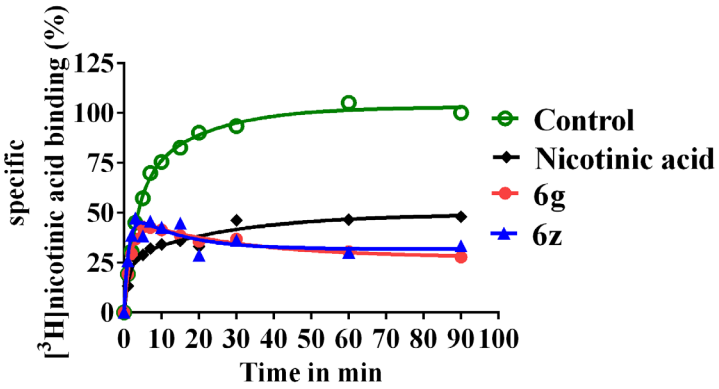


Fig. 4. [^{3}H]nicotinic acid competition association assay in the absence of ligand (control) and in the presence of 1 × IC_{50} of unlabeled **nicotinic acid**, **6g** and **6z** yield their kinetic profiles. Representative graphs from one experiment performed in duplicate.

Discussion

The affinities of compounds **5c, e, i–n, r** have been described previously in the broad range of 1 nM–15 μM[37]. In the present study we report new findings on the SAR and SKR of anthranilic acid-derived agonists for the HCA_2 receptor. The mono-substituted compounds with 4-OH (**6g**)[35, 38], 2-Cl **(5o**), 2-F (**5p**) and 2-OH (**6r**) were not significantly different with respect to affinity (75–94 nM) . This simple observation illustrates the difficulty in a typical drug discovery program to triage compounds for further development, suggesting the decision, which compound to select for further development cannot be made on the classical in vitro affinities alone. Hence, we added the additional parameter of drug-target kinetics. In this particular case we were faced with the problem that the association and dissociation characteristics of the radioligand precluded the determination of the kinetic rate constants. As the k_{obs} value (apparent association rate constant, 0.21 min^{-1}) was smaller than the dissociation rate constant (k_{off}, 0.33 min^{-1}) (Fig. 3A and B), the 'true' association rate constant (k_{on}) cannot be determined from the equation, $k_{on} = (k_{obs} - k_{off})/[radioligand]$. This problem could not be solved by changing temperature or time of incubation, the concentration of radioligand or membranes, or using cell lines with different expression levels of the $hHCA_2$ receptor (data not shown). Thus, the actual residence time values could not be determined from the competition association assay, as here both rate constants (k_{on} and k_{off}) for the radioligand are needed for the calculation. However, more indirect measures for the kinetics of unlabeled competing ligands, the so-called kinetic rate index (KRI)[34] can still be assessed in the competition association assay. Indeed, these KRI values of the different compounds were quite telling. In general, a KRI > 1.0 indicates a relatively slow dissociation from the target, while a KRI < 1.0, or in this case a KRI < 0.7 or 0.8, predicts a relatively fast dissociation rate compared to the dissociation rate of the radioligand [^{3}H]-nicotinic acid[34, 39]. The 4-OH derivative **6g** had the longest residence time, displaying a KRI value of 1.31 ± 0.06 ($p < 0.005$) compared to approx. 0.7–0.9 in the case of the similar-affinity compounds, 2-Cl **(5o**), 2-F (**5p**) and 2-OH (**6r**).

Di-substituted compounds bearing the 4-OH group lacked a longer

residence time, although yielding higher affinity compounds including the 2-Cl, 4-OH derivative (**6w**). This latter disubstitution pattern has often been employed by the Merck group, although clinical candidate MK6892 has a slightly different heterocyclic 4-hydroxy-2-pyridine moiety[20, 30, 35, 36]. The tetrahydroanthranilic acid (**6z**), whose moiety is also present in MK6892[7], did have a beneficial KRI profile in our hands.

Conclusions and outlook

We investigated the structure–affinity relationships (SAR) and the structure–kinetics relationships (SKR) of a series of biphenyl anthranilic acid agonists for the HCA_2 receptor. The SAR led us to the novel 2-F, 4-OH derivative (**6x**) with an IC_{50} value of 23 ± 3 nM as the highest affinity HCA_2 agonist of the biphenyl series. However because of its low KRI value of 0.9 as an indication of the residence time, we considered it less promising.

The selection approach by combining affinity and binding kinetics resulted in two interesting 4-OH substituted lead compounds. One was the known agonist **6g** with a 3 times compromised affinity of 75 ± 5 nM compared to **6x**, but with an interesting KRI value of 1.31 ± 0.06 ($p < 0.005$) indicating a longer residence time. Also the related novel tetrahydroanthranilic acid derivative **6z** had a similar KRI value of 1.23 ± 0.03 ($p < 0.005$) and an IC_{50} value of 108 ± 4 nM. On the other hand, the thiophene bioisostere of anthranilic acid **6aa** was also instructive to us, as it showed that not all 4-OH-biphenyl ligands possess a longer residence time for the HCA_2 receptor.

Chemistry

All solvents and reagents were purchased from commercial sources and were of analytical grade. Demineralized water is simply referred to as H_2O, because it was used in all cases, unless stated otherwise (i.e., brine). ^1H and ^{13}C NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^1H NMR, 400 MHz; ^{13}C NMR, 100 MHz) at ambient temperature. Chemical shifts are reported

in parts per million (ppm), are designated by δ, and are downfield to the internal standard tetramethylsilane (TMS). Coupling constants are reported in hertz and are designated as *J*. Analytical purity of the final compounds was determined by high-performance liquid chromatography (HPLC) with a Phenomenex Gemini 3 μm C18 110A column (50 × 4.6 mm, 3 μm), measuring UV absorbance at 254 nm. The sample preparation and HPLC method was as follows: 0.3–0.6 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of $CH_3CN/H_2O/t$-BuOH and eluted from the column within 15 min at a flow rate of 1.3 mL/min. The elution method was set up as follows: 1–4 min isocratic system of H_2O/CH_3CN/1% TFA in H_2O, 80:10:10, from the 4th min, a gradient was applied from 80:10:10 to 0:90:10 within 9 min, followed by 1 min of equilibration at 0:90:10 and 1 min at 80:10:10. All compounds showed a single peak at the designated retention time and are at least 95% pure. Liquid chromatography–mass spectrometry (LC–MS) analyses were performed using Thermo Finnigan Surveyor–LCQ Advantage Max LC–MS system and a Gemini C18 Phenomenex column (50 × 4.6 mm, 3 μm). The elution method was set up as follows: 1–4 min isocratic system of H_2O/CH_3CN/1% TFA in H_2O, 80:10:10, from the 4th min, a gradient was applied from 80:10:10 to 0:90:10 within 9 min, followed by 1 min of equilibration at 0:90:10 and 1 min at 80:10:10. Thin-layer chromatography (TLC) was routinely consulted to monitor the progress of reactions, using aluminum-coated Merck silica gel F^{254} plates. Purification by column chromatography was achieved by use of Grace Davison Davisil silica column material (LC60A, 30–200 μm). Microwave reactions were carried out in a Biotage Emrys™ Optimizer using sealed tubes and at a set reaction temperature. The procedure for a series of similar compounds is given as a general procedure for all within that series, annotated by the numbers of the compounds.

Compounds **2a**[40], **2c**[41], **2e**[42], **2f**[43], **3a**[44], **3c**[32], **3e**[42], **3o**[45], **5a**[30], **5c**, **5e**, **5i**, **5j**, **5l**, **5o**, **5w**, **5n**, **5r**[37] are described in literature, but have been synthesized by another approach.

General procedure for the preparation of methyl 3-(4-(substitutedphenyl) phenyl) propionates (2a, c-f, i-l, o-p, w)[30]

Methyl 3-(4-bromophenyl)propanoate[31] **1** (1.0 equiv) and the appropriate commercially available (substituted-phenyl)boronic acid (1.5 equiv) were dissolved in a mixture of dioxane:EtOH (1:1) (concentration 1mL/mmol) and 1M aqueous $NaHCO_3$ (2.0 equiv). To the solution, $Pd(PPh_3)_4$ (2.5 mol%) was added and the mixture was heated in the microwave at 100 °C for 3 h, after which TLC showed full conversion. The reaction mixture was concentrated *in vacuo*, acidified to pH = 1 using 1 M HCl (aq), extracted with EtOAc, dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography eluting with CH_2Cl_2:Pet ether (2:1) yielded the desired biphenyl esters as solids

Methyl 3-(4-(phenyl)phenyl]propanoate (2a)

Yield = 181 mg, 78%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, J = 7.6 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.42 (t, J = 8.0 Hz, 2H), 7.33 (t, J = 7.2 Hz, 1H), 7.27 (d, J = 8.4 Hz, 2H), 3.69 (s, 3H), 3.00 (t, J = 8.0 Hz, 2H), 2.67 (t, J = 7.6 Hz, 2H) ppm.

Methyl 3-(4-(4-methoxyphenyl)phenyl]propanoate (2c)

Yield = 212 mg, 76%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51-7.46 (m, 4H), 7.24 (d, J = 8.4 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 3.85 (s, 3H), 3.69 (s, 3H), 2.98 (t, J = 8.0 Hz, 2H), 2.67 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(4-chlorophenyl)phenyl)propanoate (2d)

Yield = 372 mg, 82%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51-7.47 (m, 4H), 7.40-7.37 (m, 2H), 7.28-7.25 (m, 2H), 3.68 (s, 3H), 2.99 (t, J = 7.6 Hz, 2H), 2.67 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(4-fluorophenyl)phenyl)propanoate (2e)

Yield = 337 mg, 77%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50-7.47 (m, 2H), 7.24 (d, J = 8.4 Hz, 2H), 7.25-7.21 (m, 2H), 7.10-7.05 (m, 2H), 3.65 (s, 3H), 2.97 (t, J = 7.6 Hz, 2H), 2.64 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(4-(trifluoromethyl)phenyl)phenyl)propanoate (2f)

Yield = 494 mg, 89%. ^{1}H NMR (400 MHz ,$CDCl_3$): δ 7.67 (t, J = 8.8 Hz, 4H), 7.53 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 3.69 (s, 3H), 3.01 (t, J = 7.6 Hz, 2H), 2.68 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(3-methylphenyl)phenyl)propanoate (2i)

Yield = 400 mg, 95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, J = 8.0 Hz, 2H), 7.36-7.33 (m, 2H), 7.26 (t, J = 7.6 Hz, 1H), 7.21 (d, J = 8.0 Hz, 2H), 7.12-7.09 (m, 1H), 3.62 (s, 3H), 2.94 (t, J = 7.6 Hz, 2H), 2.61 (t, J = 8.0 Hz, 2H), 2.36 (s, 3H) ppm.

Methyl 3-(4-(3-methoxyphenyl)phenyl)propanoate (2j)

Yield = 557 mg, 71%. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.51 (d, J = 8.4 Hz, 2H), 7.33 (t, J = 8.0 Hz, 1H), 7.26 (d, J = 8.0 Hz, 2H), 7.16 (d, J = 7.6 Hz, 1H), 7.10 (t, J = 2.0 Hz, 1H), 6.88 (dd, J = 8.0, 2.4 Hz, 1H), 3.85 (s, 3H), 3.68 (s, 3H), 2.99 (t, J = 8.0 Hz, 2H), 2.65 (t, J= 7.2 Hz, 2H) ppm.

Methyl 3-(4-(3-chlorophenyl)phenyl)propanoate (2k)

Yield = 393 mg, 69%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.53 (m, 1H), 7.50-7.43 (m, 3H), 7.32 (t, J = 7.6 Hz, 1H), 7.30-7.23 (m, 3H), 3.68 (s, 3H), 3.00 (t, J = 8.0 Hz, 2H), 2.67 (t, J = 8.0, 2H) ppm.

Methyl 3-(4-(3-fluorophenyl)phenyl)propanoate) (2l)

Yield = 338 mg, 79%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, J = 8.4 Hz, 2H), 7.35-7.30 (m, 2H), 7.26-7.21 (m, 3H), 7.01-6.96 (m, 1H), 3.65 (s, 3H), 2.97 (t, J = 7.6 Hz, 2H), 2.64 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(2-chlorophenyl)phenyl)propanoate (2o)

Yield = 535 mg, 93%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (dd, J = 7.6, 1.2 Hz, 1H), 7.38 (d, J = 8.0 Hz, 2H), 7.34-7.30 (m, 2H), 7.29-7.24 (m, 3H), 3.70 (s, 3H), 3.01 (t, J = 8.0 Hz, 2H), 2.69 (t, J = 8.4 Hz, 2H) ppm.

Methyl 3-(4-(2-fluorophenyl)phenyl)propanoate (2p)

Yield = 386 mg, 91%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (dd, J= 8.0, 1.2 Hz, 2H), 7.40 (td, J = 7.8, 2.0 Hz, 1H), 7.30-7.25 (m, 3H), 7.19-7.10 (m, 2H), 3.67 (s, 3H), 2.99 (t, J = 8.0 Hz, 2H), 2.66 (t, J = 8.0 Hz, 2H) ppm.

Methyl 3-(4-(2-chloro-4-methoxyphenyl)phenyl)propanoate (2w)

Yield = 718 mg, 95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (d, J = 8.0 Hz, 2H), 7.25-7.30 (m, 3H), 7.01 (d, J = 2.8 Hz, 1H), 6.86 (dd, J = 8.8, 2.8 Hz, 1H), 3.84 (s, 3H), 3.70 (s, 3H), 3.00 (t, J = 7.6 Hz, 2H), 2.69 (t, J = 8.0 Hz, 2H) ppm.

General procedure to obtain the phenyl propanoic acids (3a, c-f, i-l, o-p, w) by ester saponification

Ester **2a, c-f, i-l, o-p, w** (1.35 mmol, 1.0 eq.) was dissolved in dioxane (0.15mmol/mL) and a 5M aqueous NaOH (10.0 eq.) solution was added. After being stirred at room temperature for 3 h, the mixture was acidified, extracted with EtOAc, dried over $MgSO_4$ and concentrated *in vacuo* to obtain the carboxylic acids (**3a, c-f, i-l, o-p, w)** as solids.

3-(4-(Phenyl)phenyl)propanoic acid (3a)

Yield = 161 mg, 69%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, J = 8.0 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 7.43 (t, J = 7.6 Hz, 2H), 7.33 (t, J = 7.6 Hz, 1H), 7.29 (d, J = 8.0 Hz, 2H), 3.01 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(4-Methoxyphenyl)phenyl)propanoic acid (3c)

Yield = 172 mg, 86%. ^{1}H NMR (400 MHz, MeOD): δ 7.51 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.0 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 6.97 (d, J = 8.4 Hz, 2H), 3.82 (s, 3H), 2.93 (t, J = 8.0 Hz, 2H), 2.62 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(4-Chlorophenyl)phenyl)propanoic acid (3d)

Yield = 431 mg, quantitative. ^{1}H NMR (400 MHz, MeOD): δ 7.59-7.57 (m, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.43-7.40 (m, 2H), 7.31 (d, J = 8.0 Hz, 2H), 2.95 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 8.0 Hz, 2H) ppm.

3-(4-(4-Fluorophenyl)phenyl)propanoic acid (3e)

Yield = 366 mg, quantitative. ^{1}H NMR (400 MHz, MeOD): δ 7.61-7.57 (m, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H), 7.14 (t, J = 8.8 Hz, 2H), 2.95 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H) ppm.

3-[4-(4-(Trifluoromethyl)phenyl)phenyl]propanoic acid (3f)

Yield = 437 mg, 93%. ^{1}H NMR (400 MHz, MeOD): δ 7.79 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 2.97 (t, J = 7.6 Hz, 2H), 2.65 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(3-Methylphenyl)phenyl)propanoic acid (3i)

Yield = 471 mg, quantitative. ^{1}H NMR (400 MHz, MeOD): δ 7.17 (d, J = 8.0 Hz, 2H), 7.07-7.03 (m, 2H), 6.97-6.92 (m, 3H), 6.79 (d, J = 7.2 Hz, 1H), 2.63 (t, J = 7.6 Hz, 2H), 2.32 (t, J = 8.0 Hz, 2H), 2.04 (s, 3H) ppm.

3-(4-(3-Methoxyphenyl)phenyl)propanoic acid (3j)

Yield = 473 mg, 90%. ^{1}H NMR (MeOD, 400 MHz): δ 7.52 (d, J = 8.0 Hz, 2H), 7.32-7.28 (m, 3H), 7.16 (dd, J = 7.6, 0.8 Hz, 1H), 7.11 (t, J = 2.4 Hz, 1H), 6.89-6.87 (m, 1H), 3.83 (s, 3H), 2.95 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(3-Chlorophenyl)phenyl)propanoic acid (3k)

Yield = 357 mg, 86%. ^{1}H NMR (400 MHz, MeOD): δ 7.59 (t, J = 2.0 Hz, 1H), 7.54-7.51 (m, 3H), 7.40 (t, J = 7.6 Hz, 1H), 7.34-7.31 (m, 5H), 2.96 (t, J = 7.6 Hz, 2H), 2.64 (t, J = 8.0 Hz, 2H) ppm.

3-(4-(3-Fluorophenyl)phenyl)propanoic acid (3l)

Yield = 319 mg, 99%. ^{1}H NMR (400 MHz, MeOD): δ 7.53 (d, J = 8.0 Hz, 2H), 7.45-7.38 (m, 2H), 7.31 (d, J = 8.4 Hz, 3H), 7.06-7.00 (m, 1H), 2.95 (t, J = 7.6 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(2-Chlorophenyl)phenyl)propanoic acid (3o)

Yield = 496 mg, quantitative. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45 (d, J = 7.2 Hz, 1H), 7.37 (d, J =

8.0 Hz, 2H), 7.33-7.23 (m, 5H), 3.02 (t, J = 7.6 Hz, 2H), 2.74 (t, J = 8.0 Hz, 2H) ppm.

3-(4-(2-Fluorophenyl)phenyl)propanoic acid (3p)

Yield = 349 mg, 95%. ^{1}H NMR (400 MHz, MeOD): δ 7.47-7.42 (m, 3H), 7.36-7.30 (m, 3H), 7.22 (td, J = 7.6, 1.2 Hz, 1H), 7.18-7.13 (m, 1H), 2.96 (t, J = 7.6 Hz, 2H), 2.65 (t, J = 7.6 Hz, 2H) ppm.

3-(4-(2-Chloro-4-methoxyphenyl)phenyl)propanoic acid (3w)

Yield = 653 mg, 95%. 1H NMR (400 MHz, MeOD): δ 7.30-7.23 (m, 5H), 7.04 (d, J = 2.8 Hz, 1H), 6.92 (dd, J = 8.4, 2.4 Hz, 1H), 3.82 (s, 3H), 2.95 (t, J = 7.6 Hz, 2H), 2.64 (t, J = 8.0 Hz, 2H) ppm.

General procedure of the preparation of amides (4a, c-f, i-l, o-p, w) by the use EDCI*HCl

The respective biphenyl propionic acid (**3a, c-f, i-l, o-p, w**) (1.4 eq.), methyl-2-amino-benzoate (1.0 eq.), EDCI*HCl (2.0 eq.) and DMAP (0.1 eq.) were dissolved in dry CH_2Cl_2 and this solution was stirred at room temperature for 72 h under nitrogen. The reaction mixture was adsorbed on silica and purification by column chromatography (Pet. ether/EtOAc (9:1) or CH_2Cl_2/Pet. ether (1:1) to 100% CH_2Cl_2) was performed, which was followed by recrystallization from EtOAc.

Methyl 2-(3-(4-phenylphenyl)propanamido)benzoate (4a)

Pet. ether/EtOAc (9:1). Yield = 149 mg, 70%). 1H NMR (400 MHz, $CDCl_3$): δ 11.10 (s, 1H), 7.58-7.51 (m, 5H), 7.44-7.41 (m, 2H), 7.34-7.32 (m, 3H), 7.08 (t, J = 8.0 Hz, 1H), 3.89 (s, 3H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(4-methoxyphenyl)phenyl)propanamido)benzoate (4c)

Pet. ether/EtOAc (9:1). Yield = 72 mg, 27%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.09 (s, 1H), 8.73 (d, J = 8.0 Hz, 1H), 8.03 (d, J = 1.6 Hz, 1H), 7.55-7.47 (m, 5H), 7.31 (d, J = 8.0 Hz, 2H), 7.08 (t, J = 7.2 Hz, 1H), 3.89 (s, 3H), 3.85 (s, 3H), 3.11 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(4-chlorophenyl)phenyl)propanamido)benzoate (4d)

Pet. ether/EtOAc (9:1). Yield = 98 mg, 15%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.10 (s, 1H), 8.73 (d, J = 8.4 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 7.54 (t, J = 7.6 Hz, 1H), 7.49-7.58 (m, 4H), 7.37 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.07 (t, J = 7.6 Hz, 1H), 3.88 (s, 3H), 3.12 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(4-fluorophenyl)phenyl)propanamido)benzoate (4e)

Pet. ether/EtOAc (9:1). Yield = 95 mg, 19%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.09 (s, 1H), 8.74 (dd, J = 8.4, 0.8 Hz, 1H), 8.00 (dd, J = 8.0, 1.6 Hz, 1H), 7.56-7.48 (m, 3H), 7.46 (d, J = 8.0 Hz, 2H), 7.12-7.05 (m, 3H), 3.88 (s, 3H), 3.12 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(4-trifluoromethylphenyl)phenyl)propanamido)benzoate (4f)

CH_2Cl_2/Pet. ether (1:1). Yield = 170 mg, 27%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.11 (s, 1H), 8.74 (d, J = 8.4 Hz, 1H), 8.02 (d, J = 8.0 Hz, 1H), 7.69-7.65 (m, 4H), 7.57-7.52 (m, 3H), 7.37 (d, J = 8.0 Hz, 2H), 7.08 (t, J = 7.6 Hz, 1H), 3.90 (s, 3H), 3.14 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(3-methylphenyl)phenyl)propanamido)benzoate (4i)

Pet. ether/EtOAc (9:1). Yield = 127 mg, 22%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.09 (s, 1H), 8.74 (d, J = 8.0 Hz, 1H), 7.99 (dd, J = 8.0, 1.6 Hz, 1H), 7.55-7.49 (m, 3H), 7.36 (d, J = 8.4 Hz, 2H), 7.12-7.28 (m, 3H), 7.13 (d, J = 7.2 Hz, 1H), 7.05 (t, J = 7.2 Hz, 1H), 3.87 (s, 3H), 3.11 (t, J = 7.6 Hz, 2H), 2.78 (t, J = 8.0 Hz, 2H), 2.40 (s, 3H) ppm.

Methyl 2-(3-(4-(3-methoxyphenyl)phenyl)propanamido)benzoate (4j)

CH_2Cl_2/Pet.ether (1:1). Yield = 249 mg, 35%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.09 (s, 1H), 8.74 (d, J = 8.4 Hz, 1H), 8.01 (dd, J = 8.0, 1.6 Hz, 1H), 7.56-7.50 (m, 3H), 7.35-7.31 (m, 3H), 7.15 (d, J = 7.6 Hz, 1H), 7.10-7.05 (m, 2H), 6.87 (dd, J= 8.4, 2.4 Hz, 1H), 3.88 (s, 3H), 3.85 (s, 3H), 3.12 (t, J= 7.6 Hz, 2H), 2.79 (t, J= 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(3-chlorophenyl)phenyl)propanamido)benzoate (4k)

Pet. ether/EtOAc (9:1) and a second column was performed with CH_2Cl_2/Pet.ether (2:1). Yield = 83 mg, 15%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.10 (s, 1H), 8.73 (d, J = 8.4 Hz, 1H), 8.02 (d, J = 8.0 Hz, 1H), 7.57-7.43 (m, 5H), 7.35-7.26 (m, 4H), 7.08 (t, J = 7.6 Hz, 1H), 3.90 (s, 3H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(3-fluorophenyl)phenyl)propanamido)benzoate (4l)

CH_2Cl_2/Pet. ether (1:1) to 100% CH_2Cl_2. Yield = 15 mg, 6%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.10 (s, 1H), 8.73 (d, J = 8.4 Hz, 1H), 8.01 (dd, J = 8.0, 1.6 Hz, 1H), 7.57-7.49 (m, 3H), 7.40-7.33 (m, 5H), 7.27-7.25 (m, 1H), 7.10-6.99 (m, 2H), 3.89 (s, 3H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4-(2-chlorophenyl)phenyl)propanamido)benzoate (4o)

DCM/Pet. ether (1:1) to 100% CH_2Cl_2. Yield = 245 mg, 33%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.23 (s, 1H), 8.75 (d, J = 8.4, 1H), 7.98 (dd, J = 8.0, 1.2 Hz, 1H), 7.51 (t, J = 7.6 Hz, 1H), 7.42 (d, J = 8.4 Hz, 1H), 3.36 (d, J = 8.0 Hz, 2H), 7.32-7.19 (m, 5H), 7.03 (t, J = 8.0 Hz, 1H), 3.85 (s, 3H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.4 Hz, 2H) ppm.

Methyl 2-(3-(4-(2-fluorophenyl)phenyl)propanamido)benzoate (4p)

CH_2Cl_2/Pet. ether (1:1) to 100% CH_2Cl_2. Yield = 176 mg, 33%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.11 (s, 1H), 8.74 (d, J = 8.4 Hz, 1H), 7.98 (dd, J = 6.8, 1.6 Hz, 1H), 7.54-7.46 (m, 3H), 7.39 (td, J = 7.6, 1.6 Hz, 1H), 7.32 (d, J = 8.4 Hz, 2H), 7.29-7.22 (m, 1H), 7.18-7.02 (m, 3H), 3.86 (s, 3H), 3.12 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 8.4 Hz, 2H) ppm.

Methyl 2-(3-(4-(2-chloro-4-methoxyphenyl)phenyl)propanamido)benzoate (4w)

CH_2Cl_2/Pet. ether (1:1) to 100% CH_2Cl_2. Yield = 298 mg, 31%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.12 (s, 1H), 8.74 (dd, J =8.4, 0.8 Hz, 1H), 8.02 (dd, J =8.0, 1.6 Hz, 1H), 7.55 (td, J = 8.0, 0.8 Hz, 1H), 7.36-7.30 (m, 4H), 7.23 (d, J = 8.4 Hz, 1H), 7.08 (td, J = 6.8, 1.2 Hz, 1H), 7.01 (d, J = 2.8 Hz, 1H), 6.85 (dd, J = 8.8, 2.8 Hz, 1H), 3.91 (s, 3H), 3.83 (s, 3H), 3.13 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 8.4 Hz, 2H) ppm.

Methyl 2-(3-(4-(4-methoxyphenyl)phenyl)propanamido)cyclohex-1-ene-1-carboxylate (4z)

Acid 3c (75 mg, 0.30 mmol, 1.0 eq.) was refluxed in $SOCl_2$ (10 mL) for 1.5 h under a N_2 atmosphere. The excess of $SOCl_2$ was evaporated (and co-evaporated with toluene twice) and the crude mixture was dissolved in toluene[30]. Methyl 2-amino-1-cyclohexene-1-carboxylate[20] (68 mg, 0.44 mmol, 1.5 eq.) was added and the mixture and was stirred overnight at 70 °C under a N_2 atmosphere. Upon completion of the reaction, the reaction mixture was filtered and the filtrate was concentrated. The filtrate was purified by column chromatography (Pet. ether/EtOAc 2:1) to yield 20 mg, 17% of the target compound 4z as a solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.60 (s, 1H), 7.59-7.44 (m, 5H), 7.28 (t, J = 7.6 Hz, 1H), 6.99-6.95 (m, 2H), 3.84 (s, 3H), 3.71 (s, 3H), 3.04-2.97 (m, 4H), 2.67 (t, J = 8.0 Hz, 2H), 2.29 (t, J = 8.0 Hz, 2H), 1.66-1.55 (m, 4H) ppm.

General procedure to yield the substituted anthranilic acids (5a, c-f, i-l, o-p, w, z, aa) by saponification

Anthranilic ester **4a, c-f, i-l, o-p, w, z, aa** (98 mg, 0.25 mmol) was dissolved in dioxane (0.1 mmol/mL) and NaOH (5M aq.) (10 eq.) was added. After being stirred at RT for 24 h, the reaction mixture was acidified to pH = 1 (1M HCl (aq.)), extracted with EtOAc, dried over $MgSO_4$ and the volatiles were evaporated in vacuo to yield the final anthranilic acids **5a, c-f, i-l ,o-p, w** and both the cyclohexene **5z** and thiophene analogues **5aa** as the pure solids.

2-(3-(4-Phenylphenyl)propanamido)benzoic acid (5a)

Yield = 16 mg, 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.86 (s, 1H), 8.76 (d, J = 8.8 Hz, 1H), 8.09 (d, J = 8.0 Hz, 1H), 7.61 (t, J = 7.2 Hz, 1H), 7.56-7.51 (m, 4H), 7.40 (t, J = 7.6 Hz, 2H), 7.34-7.29 (m, 3H), 7.12 (t, J = 7.6 Hz, 1H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 10.16 min. purity 95%. ESI-MS: 345.93 $[M+H]^+$.

2-(3-(4-(4-Methoxyphenyl)phenyl)propanamido)benzoic acid (5c)

Yield = 52 mg, 75%. ^{1}H NMR (400 MHz, $CDCl_3$ + MeOD): **δ** 8.62 (d, J = 8.4 Hz, 1H), 8.09 (d, J = 7.6 Hz, 1H), 7.58-7.48 (m, 5H), 7.31 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.6 Hz, 1H), 6.97 (d, J = 8.8 Hz, 2H), 3.85 (s, 3H), 3.10 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 10.09 min. purity 99%. ESI-MS: 376.00 $[M+H]^+$.

2-(3-(4-(4-Chlorophenyl)phenyl)propanamido)benzoic acid (5d)

Yield = 102 mg, 100%. Mp 196 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.86 (s, 1H), 8.75 (d, J = 8.4 Hz, 1H), 8.09 (dd, J = 8.0, 1.2 Hz, 1H), 7.60 (t, J = 8.4 Hz, 1H), 7.49-7.47 (m, 4H), 7.38 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.11 (t, J = 7.2 Hz, 1H), 3.12 (t, J = 7.6 Hz, 2H), 2.78 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 10.72 min. purity 98%. ESI-MS: 379.93 $[M+H]^+$.

2-(3-(4-(4-Fluorophenyl)phenyl)propanamido)benzoic acid (5e)

Yield = 88 mg, 97%. ^{1}H NMR (400 MHz, MeOD): δ 8.55 (d, J = 8.4 Hz, 1H), 8.06 (dd, J = 8.0, 1.6 Hz, 1H), 7.58-7.52 (m, 3H), 7.49 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.15-7.10 (m, 3H), 3.08 (t, J = 7.6 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.24 min. purity 100%. ESI-MS: 363.93 $[M+H]^+$.

2-(3-(4-(4-Trifluoromethylphenyl)phenyl)propanamido)benzoic acid (5f)

Yield = 150 mg, 91%. Mp 209 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.77 (d, J = 8.0 Hz, 2H), 7.70 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.55 (t, J = 7.2 Hz, 1H), 7.38 (d, J = 8.4 Hz, 2H), 7.13 (t, J = 7.2 Hz, 1H), 3.10 (t, J = 7.6 Hz, 2H), 2.79 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.85 min. purity 99%. ESI-MS: 413.93 $[M+H]^+$.

2-(3-(4-(3-Methylphenyl)phenyl)propanamido)benzoic acid (5i)

Yield = 122 mg, 100%. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 10.88 (s, 1H), 8.75 (d, J = 8.4 Hz, 1H), 8.09 (d, J = 8.0 Hz, 1H), 7.60 (t, J = 7.2 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 7.6 Hz, 2H), 7.30 (d, J = 8.4 Hz, 3H), 7.11 (t, J = 8.4 Hz, 2H), 3.12 (t, J = 7.2 Hz, 2H), 2.79 (t, J = 8.0, 2H), 2.39 (s, 3H) ppm. ^{13}C NMR (400 MHz, $CDCl_3$): **δ** 172.5, 171.7, 142.0, 140.9, 139.5, 138.4, 135.8, 131.9, 128.9, 128.7, 128.0, 127.9, 127.4, 124.2, 123.0, 120.8, 114.1, 40.5, 31.3, 21.6 ppm. HPLC Rt = 10.53 min. purity 100%. ESI-MS: 359.93 $[M+H]^+$.

2-(3-(4-(3-Methoxyphenyl)phenyl)propanamido)benzoic acid (5j)

After 3 days no conversion was shown by TLC. Added 10 eq. of LiOH (aq.) and refluxed for two hours, after which completion of the reaction was confirmed by TLC. Yield = 221 mg, 95%. ^{1}H NMR (400 MHz, MeOD): **δ** 8.55 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 7.2 Hz, 1H), 7.54-7.50 (m, 3H), 7.33-7.28 (m, 3H), 7.14-7.09 (m, 3H), 6.86 (dd, J = 8.4, 2.0 Hz, 1H), 3.83 (s, 3H), 3.07 (t, J = 7.6 Hz, 2H), 2.76 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.12 min. purity 98%. ESI-MS: 375.87 $[M+H]^+$.

2-(3-(4-(3-Chlorophenyl)phenyl)propanamido)benzoic acid (5k)

Yield = 55 mg, 69%. Mp 157 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.55 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 7.6 Hz, 1H), 7.58-7.50 (m, 5H), 7.41-7.30 (m, 4H), 7.13 (t, J = 7.6 Hz, 1H), 3.09 (t, J = 7.6 Hz, 2H), 2.78 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.71 min. purity 97%. ESI-MS: 379.93 $[M+H]^+$.

2-(3-(4-(3-Fluorophenyl)phenyl)propanamido)benzoic acid (5l)

Yield = 7 mg, 48%. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 10.86 (s, 1H), 8.75 (d, J = 8.8 Hz, 1H), 8.10 (d,

J = 8.0 Hz, 1H), 7.61 (t, J = 8.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.37-7.31 (m, 4H), 7.26-7.23 (m, 1H), 7.13 (t, J = 8.0 Hz, 1H), 7.01-6.97 (m, 1H), 3.13 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.27 min. purity 100%. ESI-MS: 363.93 $[M+H]^+$.

2-(3-(4-(2-Chlorophenyl)phenyl)propanamido)benzoic acid (5o)

Yield = 217 mg, 92%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.07 (s, 1H), 8.76 (d, J = 8.4 Hz, 1H), 8.11 (dd, J = 8.0, 1.2, 1H), 7.59 (td, J = 8.0, 1.6 Hz, 1H), 7.43 (dd, J = 7.2, 1.2 Hz, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.4 Hz, 2H), 7.30-7.20 (m, 3H), 7.11 (td, J = 8.0, 0.8 Hz, 1H), 3.15 (t, J = 7.2 Hz, 2H), 2.84 (t, J = 8.4 Hz, 2H) ppm. HPLC Rt = 10.42 min. purity 97%. ESI-MS: 379.93 $[M+H]^+$.

2-(3-(4-(2-Fluorophenyl)phenyl)propanamido)benzoic acid (5p)

Yield = 172 mg, 99%. Mp 162 °C. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 10.87 (s, 1H), 8.76 (d, J = 8.4 Hz, 1H), 8.11 (d, J = 8.0 Hz, 1H), 7.61 (t, J = 7.6 Hz, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.39 (t, J = 7.6 Hz, 1H), 7.34 (d, J = 8.0 Hz, 2H), 7.29-7.24 (m, 1H), 7.18-7.08 (m, 3H), 3.14 (t, J = 7.6 Hz, 2H), 2.82 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 10.17 min. purity 98%. ESI-MS: 363.93 $[M+H]^+$.

2-(3-(4-(2-Chloro-4-methoxyphenyl)phenyl)propanamido)benzoic acid (5w)

Yield = 276 mg, 96%. ^{1}H NMR (400 MHz, DMSO): δ 13.58 (s, 1H), 11.16 (s, 1H), 8.49 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.60-7.56 (m, 5H), 7.16-7.12 (m, 2H), 6.99 (d, J = 8.4 Hz, 1H), 3.81 (s, 3H), 2.99 (t, J = 7.2 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 10.42 min. purity 97%

2-(3-(4-(4-Methoxyphenyl)phenyl)propanamido)cyclohex-1-ene-1-carboxylic acid (5z)

The product was crystallized from MeOH. Yield = 8 mg, 16%. ^{1}H NMR (400 MHz, MeOD): **δ** 7.58-7.46 (m, 4H), 7.28-7.25 (m, 2H), 6.97 (d, J = 8.8, 1.2 Hz, 2H), 3.84 (s, 3H), 3.03-2.99 (m, 4H), 2.66 (t, J = 8.0 Hz, 2H), 2.35 (t, J = 8.0 Hz, 2H), 1.65-1.59 (m, 4H) ppm. HPLC Rt = 10.09 min. purity 99%

2-(3-(4′-Methoxy-[1,1′-biphenyl]-4-yl)propanamido)thiophene-3-carboxylic acid (5aa)

Yield = 19 mg, 100%. ^{1}H NMR (400 MHz, $CDCl_3$) **δ** 10.67 (s, 1H), 7.49-7.45 (m, 4H), 7.29 (d, J = 8.0 Hz, 2H), 7.23 (d, J = 5.6 Hz, 1H), 6.98-6.91 (m, 2H), 6.76 (d, J = 6.0 Hz, 1H), 3.82 (s, 3H), 3.12 (t, J = 7.6 Hz, 2H), 2.86 (t, J = 8.0 Hz, 2H) ppm.

General Suzuki coupling procedure to yield products (5b, h, n, q-v, x, y and 4aa)

Slightly modified experimental procedure of general procedure **2a-f ,i, l, o-p, w**. Instead of $Pd(PPh_3)_4$ (2.5 mol%) as the catalyst $Pd(OAc)_2$ (0.1 eq.) and PPh_3 (0.3 eq.) were used. Next to this more $NaHCO_3$ (6 eq. 1M solution) was used. This gave better yields compared to the commercial available $Pd(PPh_3)_4$ and immediately the carboxylic acid instead of the ester was

obtained. Started from iodide **8a** or **8b** (1.0 eq.) and the respective commercially available phenyl boronic acids. Purified by column chromatography using Pet. ether: EtOAc (9:1) to EtOAc.

2-(3-(4-(4-Methylphenyl)phenyl)propanamido)benzoic acid (5b)

Yield = 73 mg, 70%. Mp 187 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 10.85 (s, 1H), 8.76 (d, J = 8.4 Hz, 1H), 8.09 (d, J = 8.0, 1H), 7.61 (t, J = 8.4 Hz, 1H), 7.50 (d, J = 8.4 Hz, 2H), 7.45 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 7.6 Hz, 2H), 7.12 (t, J = 8.0 Hz, 1H), 3.12 (t, J = 7.2 Hz, 2H), 2.80 (t, J = 8.0, 2H), 2.37 (s, 3H) ppm. HPLC Rt = 10.00 min. purity 98%. ESI-MS: 360.00 $[M+H]^+$.

2-(3-(4-(4-Hydroxymethylphenyl)phenyl)propanamido)benzoic acid (5h)

Yield = 10 mg, 9%. Mp 182 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.4 Hz, 1H), 8.06 (dd, J = 8.0, 1.2 Hz, 1H), 7.57-7.52 (m, 5H), 7.40 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 7.13 (td, J = 7.8, 1.2 Hz, 1H), 4.63 (s, 2H), 3.08 (t, J = 8.0 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 8.74 min. purity 99%. ESI-MS: 376.00 $[M+H]^+$.

2-(3-(4-(2-Methylphenyl)phenyl)propanamido)benzoic acid (5n)

Yield = 58 mg, 32%. ^{1}H NMR (400 MHz, MeOD): **δ** 8.49 (d, J = 8.4 Hz, 1H), 8.04 (dd, J = 8.0, 1.6 Hz, 1H), 7.38-7.32 (m, 3H), 7.21-7.12 (m, 6H), 7.04 (td, J = 8.0, 1.2 Hz, 1H), 3.10 (t, J = 7.8 Hz, 2H), 2.77 (t, J = 7.8, 2H), 2.19 (s, 3H) ppm. HPLC Rt = 5.73 min. purity 95%. ESI-MS: 359.93 $[M+H]^+$.

2-(3-(4-(2-Trifluoromethylphenyl)phenyl)propanamido)benzoic acid (5q)

Yield = 14 mg, 7%. Mp 168 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.0 Hz, 1H), 8.07 (dd, J = 8.0, 1.2 Hz, 1H), 7.74 (d, J = 7.6 Hz, 1H), 7.60-7.51 (m, 3H), 7.33-7.29 (m, 3H), 7.21 (d, J = 8.0 Hz, 2H), 7.14 (t, J = 7.6 Hz, 1H), 3.10 (t, J = 8.0 Hz, 2H), 2.79 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 5.58 min. purity 100%. ESI-MS: 413.93 $[M+H]^+$.

2-(3-(4-(2-Methoxyphenyl)phenyl)propanamido)benzoic acid (5r)

Yield = 40 mg, 21%. 1H NMR (400 MHz, $CDCl_3$): δ 10.80 (s, 1H), 8.74 (d, J = 8.4 Hz, 1H), 8.09 (dd, J = 8.0, 1.2 Hz, 1H), 7.60 (dt, J = 7.8, 1.2 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.30-7.27 (m, 4H), 7.11 (t, J = 8.0 Hz, 1H), 7.02-6.95 (m, 2H), 3.80 (s, 3H), 3.12 (t, J = 8.0 Hz, 2H), 2.81 (t, J = 8.0 Hz, 2H) ppm.

2-(3-(4-(4-Methoxy-3-methylphenyl)phenyl)propanamido)benzoic acid (5s)

Yield = 11 mg, 10%. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.0 Hz, 1H), 8.07 (dd, J = 8.0, 1.6 Hz, 1H), 7.54 (td, J = 7.2, 1.6 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.37 (dd, J = 10.8, 2.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.13 (td, J = 7.6, 1.2 Hz, 1H), 6.93 (d, J = 8.4 Hz, 1H), 3.85 (s, 3H), 3.06 (t, J = 8.0 Hz, 2H), 2.76 (t, J = 8.0 Hz, 2H), 2.23 (s, 3H) ppm.

2-(3-(4-(3-Chloro-4-methoxyphenyl)phenyl)propanamido)benzoic acid (5t)

Yield = 18 mg, yield 36%. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 11.25 (s, 1H), 8.73 (d, J = 8.4 Hz, 1H),

8.08 (dd, J = 8.0, 1.6 Hz, 1H), 7.50-7.40 (m, 5H), 7.30 (d, J = 8.4 Hz, 2H), 7.08 (t, J = 7.2, 1H), 6.96 (d, J = 8.4 Hz, 1H), 3.93 (s, 3H), 3.10 (t, J = 8.0 Hz, 2H), 2.77 (t, J = 8.4 Hz, 2H) ppm.

2-(3-(4-(3-Fluoro-4-methoxyphenyl)phenyl)propanamido)benzoic acid (5u)

Yield = 36 mg, 30%. ^{1}H NMR (400 MHz, $CDCl_3$ + MeOD): **δ** 8.68 (d, J = 8.0 Hz, 1H), 8.08 (dd, J = 8.0, 1.6 Hz, 1H), 7.55 (td, J = 8.6, 1.6 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.33-7.27 (m, 5H), 7.10 (td, J = 7.8, 1.2 Hz, 1H), 3.93 (s, 3H), 3.10 (t, J = 8.0 Hz, 2H), 2.78 (t, J = 8.0 Hz, 2H) ppm.

2-(3-(4-(4-Methoxy-2-methylphenyl)phenyl)propanamido)benzoic acid (5v)

Yield = 15 mg, 13%. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.4 Hz, 1H), 8.07 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.28 (d, J = 8.0 Hz, 2H), 7.19-7.10 (m, 3H), 7.05 (d, J = 8.4 Hz, 1H), 6.79-6.75 (m, 2H), 3.79 (s, 3H), 3.07 (t, J = 8.0 Hz, 2H), 2.77 (t, J = 8.0 Hz, 2H), 2.16 (s, 3H) ppm.

2-(3-(4-(2-Fluoro-4-methoxyphenyl)phenyl)propanamido)benzoic acid (5x)

Yield = 19 mg, 10%. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 10.99 (s, 1H), 8.75 (d, J = 8.4 Hz, 1H), 8.08 (d, J = 8.0, 1.2 Hz, 1H), 7.57-7.54 (m, 1H), 7.48-7.45 (m, 2H), 7.34-7.27 (m, 3H), 7.12 (t, J = 7.2 Hz, 1H), 6.76-6.67 (m, 2H), 3.35 (s, 3H), 3.13 (t, J = 7.2 Hz, 2H), 2.78 (t, J = 7.2 Hz, 2H) ppm.

2-(3-(4-(2,4-Dimethoxyphenyl)phenyl)propanamido)benzoic acid (5y)

Yield = 30 mg, 15%. ^{1}H NMR (400 MHz, $CDCl_3$ + MeOD): **δ** 11.53 (s, 1H), 8.62 (d, J = 8.4 Hz, 1H), 8.09 (dd, J = 8.0, 1.6 Hz, 1H), 7.55 (td, J = 8.0, 1.6 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 7.19 (d, J = 9.2 Hz, 1H), 7.22 (t, J = 8.0 Hz, 1H), 6.58-6.56 (m, 2H), 3.85 (s, 3H), 7.77 (s, 3H), 3.08 (t, J = 8.0 Hz, 2H), 2.79 (t, J = 8.0 Hz, 2H) ppm.

Methyl 2-(3-(4'-methoxy-[1,1'-biphenyl]-4-yl)propanamido)thiophene-3-carboxylate (4aa)

Purified by column chromatography using Pet. ether/EtOAc (4:1) yielding 20 mg, 23% as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** 10.97 (s, 1H), 7.49 (t, J = 8.0 Hz, 4H), 7.27 (t, J = 8.0 Hz, 2H), 7.18 (d, J = 5.6 Hz, 1H), 6.96 (d, J = 8.8 Hz, 2H), 6.73 (d, J = 6.0 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.12 (t, J = 8.0 Hz, 2H), 2.84 (t, J = 8.0 Hz, 2H) ppm.

General demethylation procedure by BBr_3 (6g, m, r-aa)

Demethylation of methoxyphenyl compounds (**5c, j, r-aa**) was performed following the protocol described in the patent of Daiichi Sankyo Company, Limited Chuo-ku[32]. The methoxyphenyl compounds **5c, j, r-aa** (1.0 eq.) were dissolved in dry CH_2Cl_2 (0.05 mmol/mL) and stirred at -78 °C under a nitrogen atmosphere. A 1M BBr_3 in CH_2Cl_2 (5.0 eq.) solution was slowly added drop wise and after the addition was completed the mixture was allowed to reach RT and stirring was continued for 4 h. The mixture was cooled to -78 °C and water was added. At RT the precipitated product was collected by filtration and washed with water and CH_2Cl_2 to obtain the pure hydroxyphenyl products **6g, m, r-aa** as solids.

2-(3-(4-(4-Hydroxyphenyl)phenyl)propanamido)benzoic acid (6g)[37]
Yield = 24 mg, 55%. ^{1}H NMR (400 MHz, MeOD): δ 8.56 (d, J = 8.4 Hz., 1H), 8.06 (d, J = 7.6 Hz, 1H), 7.55 (t, J = 7.6 Hz, 1H), 7.44 (d, J = 8.0 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 7.13 (t, J = 7.6 Hz, 1H), 6.82 (d, J = 8.4 Hz, 2H), 3.05 (t, J = 7.6 Hz, 2H), 3.75 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 9.00 min. purity 97%. ESI-MS: 361.93 $[M+H]^+$.

2-(3-(4-(3-Hydroxyphenyl)phenyl)propanamido)benzoic acid (6m)[37]
Yield = 61 mg, 39%. ^{1}H NMR (400 MHz, MeOD): δ 8.55 (d, J = 8.4 Hz, 1H), 8.05 (d, J = 7.6 Hz, 1H), 7.53-7.46 (m, 3H), 7.29 (d, J = 8.0 Hz, 2H), 7.20 (t, J = 7.6 Hz, 1H), 7.12 (t, J = 7.6 Hz, 1H), 7.04-6.99 (m, 2H), 6.73 (dd, J = 7.6, 1.6 Hz, 1H), 3.06 (t, J = 7.2 Hz, 2H), 2.75 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 10.08 min. purity 97%. ESI-MS: 361.93 $[M+H]^+$.

2-(3-(4-(2-Hydroxyphenyl)phenyl)propanamido)benzoic acid (6r)[37]
Yield = 22 mg, 56%. ^{1}H NMR (400 MHz, Acetone-d6): **δ** 11.29 (s, 1H), 8.76 (d, J = 8.8 Hz, 1H), 8.35 (br s, 1H), 8.09 (d, J = 7.6 Hz, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.52 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 7.6 Hz, 1H), 7.19-7.11 (m, 2H), 6.97 (d, J = 8.0 Hz, 1H), 6.90 (t, J = 7.4 Hz, 1H), 3.07 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 7.8 Hz, 2H) ppm. HPLC Rt = 9.28 min. purity 99%. ESI-MS: 361.93 $[M+H]^+$.

2-(3-(4-(4-Hydroxy-3-methylphenyl)phenyl)propanamido)benzoic acid (6s)
Yield = 7 mg, 62%. Mp 174 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.55 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.55 (td, J = 7.8, 1.2 Hz, 1H), 7.44 (d, J = 8.0 Hz, 2H), 7.29-7.25 (m, 3H), 7.22 (dd, J = 8.4, 2.0 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 3.05 (t, J = 8.0 Hz, 2H), 2.75 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 9.31 min. purity 98%. ESI-MS: 376.00 $[M+H]^+$.

2-(3-(4-(3-Chloro-4-hydroxyphenyl)phenyl)propanamido)benzoic acid (6t)
Yield = 1.0 mg, 2% . ^{1}H NMR (400 MHz, MeOD): **δ** 8.57 (d, J = 8.0 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 7.55-7.50 (m, 2H), 7.45 (d, J = 8.0 Hz, 2H), 7.35 (dd, J = 8.4, 2.4 Hz, 1H), 7.31 (d, J = 8.0 Hz, 2H), 7.13 (t, J = 7.2 Hz, 1H), 6.95 (d, J = 8.4 Hz, 1H), 3.07 (t, J = 7.6 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 9.49 min. purity 98%. ESI-MS: 395.93 $[M+H]^+$.

2-(3-(4-(3-Fluoro-4-hydroxyphenyl)phenyl)propanamido)benzoic acid (6u)
Yield = 21 mg, 59%. Mp 176 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.55 (d, J = 8.4 Hz, 1H), 8.06 (dd, J = 8.0, 1.2 Hz, 1H), 7.55 (td, J = 8.2, 1.2 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.30-7.27 (m, 3H), 7.21 (dd, J = 8.4, 1.2 Hz, 1H), 7.13 (t, J = 8.0 Hz, 1H), 6.95 (t, J = 8.8 Hz, 1H), 3.06 (t, J = 8.0 Hz, 2H), 2.76 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 9.13 min. purity 97%. ESI-MS: 380.00 $[M+H]^+$.

2-(3-(4-(4-Hydroxy-2-methylphenyl)phenyl)propanamido)benzoic acid (6v)
Yield = 14 mg, 96%. Mp 174 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.4 Hz, 1H), 8.06 (dd, J = 8.0, 1.2 Hz, 1H), 7.54 (td, J = 8.0, 1.6 Hz, 1H), 7.26 (d, J = 8.0 Hz, 2H), 7.15-7.11 (m, 3H), 6.96

(d, J = 8.4 Hz, 1H), 6.67-6.61 (m, 2H), 3.07 (t, J = 7.6 Hz, 2H), 2.76 (t, J = 8.0 Hz, 2H), 2.12 (s, 3H) ppm. HPLC Rt = 9.16 min. purity 99%. ESI-MS: 375.93 $[M+H]^+$.

2-(3-(4-(2-Chloro-4-hydroxyphenyl)phenyl)propanamido)benzoic acid (6w)[37]

Yield = 119 mg, 59%. ^{1}H NMR (400 MHz, DMSO): **δ** 11.15 (s, 1H), 9.95 (s, 1H), 8.49 (d, J = 8.4 Hz, 1H), 7.97 (dd, J = 8.0, 1.2 Hz, 1H), 7.58 (t, J = 8.4 Hz, 1H), 7.33-7.27 (m, 4H), 7.19-7.12 (m, 2H), 6.90 (s, 1H), 6.80 (dd, J = 8.4, 2.4 Hz, 1H), 2.98 (t, J = 7.2 Hz, 2H), 2.76 (t, J = 6.6 Hz, 2H) ppm. HPLC Rt = 9.36 min. purity 99%. ESI-MS: 395.93 $[M+H]^+$.

2-(3-(4-(2-Fluoro-4-hydroxyphenyl)phenyl)propanamido)benzoic acid (6x)

Yield = 6 mg, 32%. Mp 205 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.4 Hz, 1H), 8.07 (dd, J = 8.0, 1.2 Hz, 1H), 7.55 (t, J = 8.4 Hz, 1H), 7.38 (d, J = 6.8 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H), 7.24 (t, J = 8.8 Hz, 1H), 7.14 (t, J = 7.6 Hz, 1H), 6.65 (dd, J = 8.4, 2.4 Hz, 1H), 6.56 (dd, J = 12.8, 2.4 Hz, 1H), 3.07 (t, J = 8.0 Hz, 2H), 2.77 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 9.16 min. purity 98%. ESI-MS: 380.00 $[M+H]^+$.

2-(3-(4-(2,4-Dihydroxyphenyl)phenyl)propanoylamino)benzoic acid (6y)

Yield = 12 mg, 45%. Mp 162 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 8.56 (d, J = 8.0 Hz, 1H), 8.07 (dd, J = 8.0, 1.6 Hz, 1H), 7.55 (td, J = 7.2, 1.6 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 7.13 (td, J = 7.6, 1.2 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.37-6.33 (m, 2H), 3.05 (t, J = 8.0 Hz, 2H), 2.76 (t, J = 8.0 Hz, 2H) ppm. HPLC Rt = 8.34 min. purity 97%. ESI-MS: 378.07 $[M+H]^+$.

2-(3-(4-(4-Hydroxyphenyl)phenyl)propanamido)cyclohex-1-ene-1-carboxylic acid (6z)

Yield = 2 mg, 27%. ^{1}H NMR (400 MHz, MeOD): **δ** 7.47-7.41 (m, 4H), 7.25 (d, J = 8.4 Hz, 2H) , 6.83 (d, J = 8.8 Hz, 2H), 2.99 (t, J = 7.6 Hz, 2H), 2.91-2.89 (m, 2H), 2.65 (t, J = 8.0 Hz, 2H), 2.36-2.30 (m, 2H), 1.67-1.61 (m, 4H) ppm. HPLC Rt = 9.21 min. purity 97%. ESI-MS: 366.93 $[M+H]^+$.

2-(3-(4-(4-Hydroxyphenyl)phenyl)propanamido)thiophene-3-carboxylic acid (6aa)

Yield = 2 mg, 7%. Mp 199 °C. ^{1}H NMR (400 MHz, MeOD): **δ** 7.46 (d, J = 8.0 Hz, 2H), 7.41 (dt, J = 8.4, 2.0 Hz, 2H), 7.28 (d, J = 8.0 Hz, 2H), 7.19 (d, J = 6.0 Hz, 1H), 6.85-6.81 (m, 3H), 3.06 (t, J = 7.6 Hz, 2H), 2.86 (t, J = 7.6 Hz, 2H) ppm. HPLC Rt = 8.78 min. purity 98% ESI-MS: 368.13 $[M+H]^+$.

General amidation procedure to yield compounds 8a-b

3-(4-iodophenyl)propanoic acid (7)[33] (1.0 eq.) was added to $SOCl_2$ (0.2 mmol/mL) under a nitrogen atmosphere. The mixture was refluxed for 1.5 h after which the $SOCl_2$ was evaporated in vacuo. The residue was co evaporated 2 times with toluene and subsequently dissolved in toluene (0.1 mmol/mL). Methyl-2-aminobenzoate or methyl 2-aminothiophene-3-carboxylate (1.4 eq.) was added and the mixture was stirred overnight at room temperature under a nitrogen atmosphere. Upon completion of the reaction, the precipitate was filtered off and the

filtrate was concentrated. The obtained residue was purified by column chromatography (Pet Et/EtOAc 4:1) to give the target compound.

Methyl 2-(3-(4-iodophenyl)propanamido)benzoate (8a)

Started from methyl-2-aminobenzoate, yielding 1.41 g (96%) of the product as a white solid. 1H NMR (400 MHz, $CDCl_3$) δ 11.07 (s, 1H), 8.70 (d, J = 8.4 Hz, 1H), 8.02 (dd, J = 8.0, 1.6 Hz, 1H), 7.61-7.59 (m, 2H), 7.54 (dt, J = 8.0, 1.6 Hz, 1H), 7.08 (dt, J = 8.0, 1.2 Hz, 1H), 7.01 (d, J = 8.0 Hz, 2H), 3.92 (s, 3H), 3.02 (t, J = 7.6 Hz, 2H), 2.73 (t, J = 7.6 Hz, 2H) ppm.

Methyl 2-(3-(4-iodophenyl)propanamido)thiophene-3-carboxylate (8b)

Started from methyl 2-aminothiophene-3-carboxylate, Yield = 15 mg, 38%, white solid. ^{1}H NMR (400 MHz, MeOD): δ 7.60 (d, J = 8.4 Hz, 2H), 7.18 (d, J = 6.0 Hz, 1H), 7.05 (d, J = 8.0 Hz, 2H), 6.85 (d, J = 5.6 Hz, 1H), 3.87 (s, 3H), 2.99 (t, J = 8.0 Hz, 2H), 2.84 (t, J = 8.0 Hz, 2H) ppm.

Biological assays

Cell culture and membrane preparation

Human embryonic kidney (HEK293T) cells were transfected with the N-Flag-tagged HCA_2 receptor in pcDNA3.1 using a standard calcium phosphate protocol[46]. The receptor expression levels of all positive stable clones were assessed with a radioligand binding assay[47]. In all assays the HEK293T cells with highest expression level of the human HCA_2 receptor (HEK293T-hHCA_2) were used. Cell culture and membrane preparation were performed as described by us before[48]. The membrane aliquots in all assays were 35 μg of protein per well.

[^{3}H]nicotinic acid equilibrium displacement assay

Membranes of the HEK293T-hHCA_2 cell line were incubated for 1 h at 25 °C with 15 nM [^{3}H]nicotinic acid (specific activity: 50 Ci/mmol), which was obtained from Biotrend (Cologne, Germany). At first, all compounds were tested at one concentration, such that a final concentration of 10^{-5} M of the test compound was added in assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 at 25 °C). When radioligand displacement by the compounds was greater than 75%, full curves were recorded to obtain the compounds' IC_{50} values. Increasing concentrations of the test compounds in assay buffer were added by using a HP D300 digital

dispenser (Tecan Group Ltd, Männedorf, Switzerland). The total assay volume was 100 μL. To assess the total binding, a control without test compound was included. The nonspecific binding was determined in the presence of 10 μM unlabeled nicotinic acid. The final DMSO concentration in all samples was ≤ 0.25%. The incubation was terminated by rapid vacuum filtration to separate the bound and free radioligand through 96-well GF/B filter plates using a Perkin Elmer Filtermate-harvester (Perkin Elmer, Groningen, the Netherlands). Filters were subsequently washed three times with ice-cold buffer (50 mM Tris HCl, pH 7.4). The filter-bound radioactivity was determined by scintillation spectrometry using the P-E 1450 Microbeta Wallac Trilux scintillation counter (Perkin Elmer, Groningen, the Netherlands).

[^{3}H]nicotinic acid association and dissociation assays

The association binding assays were performed in a time-dependent manner by incubating membrane aliquots in a total volume of 100 μl assay buffer at 25 °C for a maximum of 120 min with 15 nM [^{3}H]-nicotinic acid. The nonspecific binding (as the 0 min time point) was determined in the presence of 10 μM unlabeled nicotinic acid. The dissociation binding assays were performed by pre-incubating membrane aliquots in a total volume of 100 μl assay buffer at 25 °C for 180 min with 15 nM [^{3}H]-nicotinic acid. After pre-incubation, dissociation was initiated by addition of 5 μl unlabeled nicotinic acid (final concentration 10 μM) for a total period of 120 min. The amounts of [^{3}H]nicotinic acid still bound to the receptor in both association and dissociation binding assays were measured at various time intervals during the incubation. Incubations were terminated and samples were harvested as described for the [^{3}H]nicotinic acid equilibrium displacement assay.

Competition association assay

The binding kinetics of unlabeled ligands were quantified using the competition association assay based on the theoretical framework by Motulsky and Mahan[49]. The competition association assay was performed in a total volume of 100 μL of assay buffer at 25 °C with 15 nM [^{3}H]nicotinic acid in the absence or presence of

$1 \times IC_{50}$ of an unlabeled competing ligand. The competition association assay was initiated by adding HEK293T-$hHCA_2$ receptor membrane aliquots at different time points with a maximum incubation time of 90 min. Incubations were terminated and samples were harvested as described for the [^{3}H]nicotinic acid equilibrium displacement assay.

Data analysis

All experimental data was analyzed by using GraphPad Prism 5.0 (GraphPad software Inc., San Diego, CA). Nonlinear regression was used to determine IC_{50} values from [^{3}H]nicotinic acid equilibrium displacement assays, and the mean IC_{50} values were obtained from three independent experiments performed in duplicate. Kinetic behavior of unlabeled competing ligands was assessed in the competition association assay, which was fitted using the *one phase exponential association* model. Kinetic rate index (KRI) values[34] were calculated by dividing the specific radioligand binding measured at t_1 = 7 min (B_{t1}) by the binding at t_2 = 90 min (B_{t2}) in the presence and absence of unlabeled competing ligands (KRI = B_{t1} / B_{t2}). Statistical evaluation (*p*-values) of KRI values was performed by a two-tailed homoscedastic Student's *t*-test using non-radioactive nicotinic acid as reference ligand; significance is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

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References

[1] Cai, T.Q., et al. *Role of GPR81 in lactate-mediated reduction of adipose lipolysis.* Biochem. Biophys. Res. Commun. (2008) 377: 987-991.

[2] Liu, C., et al. *Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81.* J. Biol. Chem. (2009) 284: 2811-2822.

[3] Taggart, A.K., et al. *(D)-β-hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G.* J. Biol. Chem. (2005) 280: 26649-26652.

[4] Ahmed, K., et al. *Deorphanization of GPR109B as a receptor for the β-oxidation intermediate 3-OH-octanoic acid and its role in the regulation of lipolysis.* J. Biol. Chem. (2009) 284: 21928-21933.

[5] Bobileva, O., et al. *Synthesis and evaluation of (E)-2-(acrylamido) cyclohex-1-enecarboxylic acid derivatives as HCA1, HCA2, and HCA3 receptor agonists.* Bioorg. Med. Chem. (2014) 22: 3654-3669.

[6] Offermanns, S., et al. *International union of basic and clinical pharmacology. LXXXII: nomenclature and classification of hydroxy-carboxylic acid receptors (GPR81, GPR109A, and GPR109B).* Pharmacol. Rev. (2011) 63: 269-290.

[7] Raghavan, S., et al. *Tetrahydro anthranilic acid as a surrogate for anthranilic acid: Application to the discovery of potent niacin receptor agonists.* Bioorg. Med. Chem. Lett. (2008) 18: 3163-3167.

[8] Tai, Y.F., et al. *Imaging microglial activation in Huntington's disease.* Brain Res. Bull. (2007) 72: 148-151.

[9] Amor, S., et al. *Inflammation in neurodegenerative diseases.* Immunology. (2010) 129: 154-169.

[10] Ahmed, K., Tunaru, S., and Offermanns, S. *GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors.* Trends Pharmacol. Sci. (2009) 30: 557-562.

[11] Lukasova, M., et al. *Nicotinic acid inhibits progression of atherosclerosis in mice through its receptor GPR109A expressed by immune cells.* J. Clin. Invest. (2011) 121: 1163.

[12] Hanson, J., Gille, A., and Offermanns, S. *Role of HCA_2 (GPR109A) in nicotinic acid and fumaric acid ester-induced effects on the skin.* Pharmacol. Ther. (2012)

[13] Gille, A., et al. *Nicotinic acid: pharmacological effects and mechanisms of action.* Annu. Rev. Pharmacol. Toxicol. (2008) 48: 79-106.

[14] Bodor, E. and Offermanns, S. *Nicotinic acid: an old drug with a promising future.* Br. J. Pharmacol. (2008) 153: S68-S75.

[15] Davidson, M.H. *Niacin use and cutaneous flushing: mechanisms and strategies for prevention.* Am. J. Cardiol. (2008) 101: S14-S19.

[16] Shen, H.C. and Colletti, S.L. *Novel patent publications on high-affinity nicotinic acid receptor agonists.* Expert Opin. Ther. Pat. (2009) 19: 957-967.

[17] Semple, G., et al. *3-(1 H-tetrazol-5-yl)-1, 4, 5, 6-tetrahydro-cyclopentapyrazole (MK-0354): a partial agonist of the nicotinic acid receptor, G-protein coupled receptor 109a, with antilipolytic but no vasodilatory activity in mice.* J. Med. Chem. (2008) 51: 5101-5108.

[18] Lai, E., et al. *Effects of a niacin receptor partial agonist, MK-0354, on plasma free fatty acids, lipids, and cutaneous flushing in humans.* J. Clin. Lipidol. (2008) 2: 375-383.

[19] Boatman, P.D., et al. *(1a R, 5a R) 1a, 3, 5, 5a-Tetrahydro-1 H-2, 3-diaza-cyclopropa [a] pentalene-4-carboxylic Acid (MK-1903): A potent GPR109a agonist that lowers free fatty acids in humans.* J. Med. Chem. (2012) 55: 3644-3666.

[20] Shen, H.C., et al. *Discovery of a biaryl cyclohexene carboxylic acid (MK-6892): a potent and selective high affinity niacin receptor full agonist with reduced flushing profiles in animals as a preclinical candidate.* J. Med. Chem. (2010) 53: 2666-70.

[21] Arrowsmith, J. and Miller, P. *Trial watch: phase II and phase III attrition rates 2011-2012.* Nat. Rev. Drug. Disc. (2013) 12: 569-569.

[22] Dahl, G. and Akerud, T. *Pharmacokinetics and the drug–target residence time concept.* Drug Discovery Today. (2013) 18: 697-707.

[23] Guo, D., et al. *Drug-target residence time—A case for G protein-coupled receptors.* Med. Res. Rev. (2014) 34: 856-892.

[24] Copeland, R.A., Pompliano, D.L., and Meek, T.D. *Drug–target residence time and its implications for lead optimization.* Nat. Rev. Drug. Disc. (2006) 5: 730-739.

[25] Swinney, D.C. *Biochemical mechanisms of new molecular entities (NMEs) approved by United States FDA during 2001-2004: mechanisms leading to optimal efficacy and safety.* Curr. top. Med. Chem. (2006) 6: 461-478.

[26] Zhang, R. and Monsma, F. *The importance of drug-target residence time.* Curr. Opin. Drug Discovery Dev. (2009) 12: 488-496.

[27] Swinney, D.C. *Biochemical mechanisms of drug action: what does it take for success?* Nat. Rev. Drug. Disc. (2004) 3: 801-808.

[28] Casarosa, P., et al. *Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs.* J. Pharmacol. Exp. Ther. (2009) 330: 660-668.

[29] Glossop, P.A., et al. *Inhalation by design: novel tertiary amine muscarinic m3 receptor antagonists with slow off-rate binding kinetics for inhaled once-daily treatment of chronic obstructive pulmonary disease.* J. Med. Chem. (2011) 54: 6888-6904.

[30] Shen, H.C., et al. *Discovery of biaryl anthranilides as full agonists for the high affinity niacin receptor.* J. Med. Chem. (2007) 50: 6303-6306.

[31] Christiansen, E., Due-Hansen, M.E., and Ulven, T. *A rapid and efficient Sonogashira protocol and improved synthesis of free fatty acid 1 (FFA1) receptor agonists.* J. Org. Chem. (2010) 75: 1301-1304.

[32] Suzuki, K. *Patent application EP2308838A1* . (2011)

[33] Cheung, S.Y., et al. *Synthesis of organometallic poly (dendrimer) s by macromonomer polymerization: effect of dendrimer size and structural rigidity on the polymerization efficiency.* Chem. Eur. J. (2009) 15: 2278-2288.

[34] Guo, D., et al. *Dual-point competition association assay A fast and high-throughput kinetic screening method for assessing ligand-receptor binding kinetics.* J. Biomol. Screen. (2013) 18: 309-320.

[35] Imbriglio, J.E., et al. *The discovery of high affinity agonists of GPR109a with reduced serum*

shift and improved ADME properties. Bioorg. Med. Chem. Lett. (2011) 21: 2721-2724.

[36] Ding, F.X., et al. *Discovery of pyrazolyl propionyl cyclohexenamide derivatives as full agonists for the high affinity niacin receptor GPR109A.* Bioorg. Med. Chem. Lett. (2010) 20: 3372-3375.

[37] Chen, W., et al., *Niacin receptor agonists, compositions containing such compounds and methods of treatment*, in *Patent application WO2006057922A2*. 2006.

[38] Schmidt, D., et al. *Anthranilic acid replacements in a niacin receptor agonist.* Bioorg. Med. Chem. Lett. (2010) 20: 3426-3430.

[39] Louvel, J., et al. *Agonists for the adenosine A1 receptor with tunable residence time. A case for nonribose 4-amino-6-aryl-5-cyano-2-thiopyrimidines.* J. Med. Chem. (2014) 57: 3213-3222.

[40] Grovenstein, E. and Cheng, Y.M. *Carbanions. XII. p-Biphenylyl migration in reactions of 1-chloro-2-p-biphenylylethane-1, 1-d2 with alkali metals.* J. Am. Chem. Soc. (1972) 94: 4971-4977.

[41] Rosen, B.M., et al. *Predicting the structure of supramolecular dendrimers via the analysis of libraries of AB3 and constitutional isomeric AB2 biphenylpropyl ether self-assembling dendrons.* J. Am. Chem. Soc. (2009) 131: 17500-17521.

[42] Viswajanani, J.S. *Patent application WO200823336A2* . (2008)

[43] Chiriano, G., et al. *A small chemical library of 2-aminoimidazole derivatives as BACE-1 inhibitors: Structure-based design, synthesis, and biological evaluation.* Eur. J. Med. Chem. (2012) 48: 206-213.

[44] Hardouin, C., et al. *Structure–activity relationships of α-ketooxazole inhibitors of fatty acid amide hydrolase.* J. Med. Chem. (2007) 50: 3359-3368.

[45] Diamond, J. *Patent application US3966801A1* . (1976)

[46] Kingston, R.E., Chen, C.A., and Rose, J.K. *Calcium phosphate transfection.* Curr. Protoc. Mol. Biol. (2003) 9.1. 1-9.1. 11.

[47] Wise, A., et al. *Molecular identification of high and low affinity receptors for nicotinic acid.* J. Biol. Chem. (2003) 278: 9869-9874.

[48] Li, Z., et al. *Effects of pyrazole partial agonists on HCA2-mediated flushing and VLDL-triglyceride levels in mice.* Br. J. Pharmacol. (2012) 167: 818-825.

[49] Motulsky, H.J. and Mahan, L. *The kinetics of competitive radioligand binding predicted by the law of mass action.* Mol. Pharmacol. (1984) 25: 1-9.

Supporting information

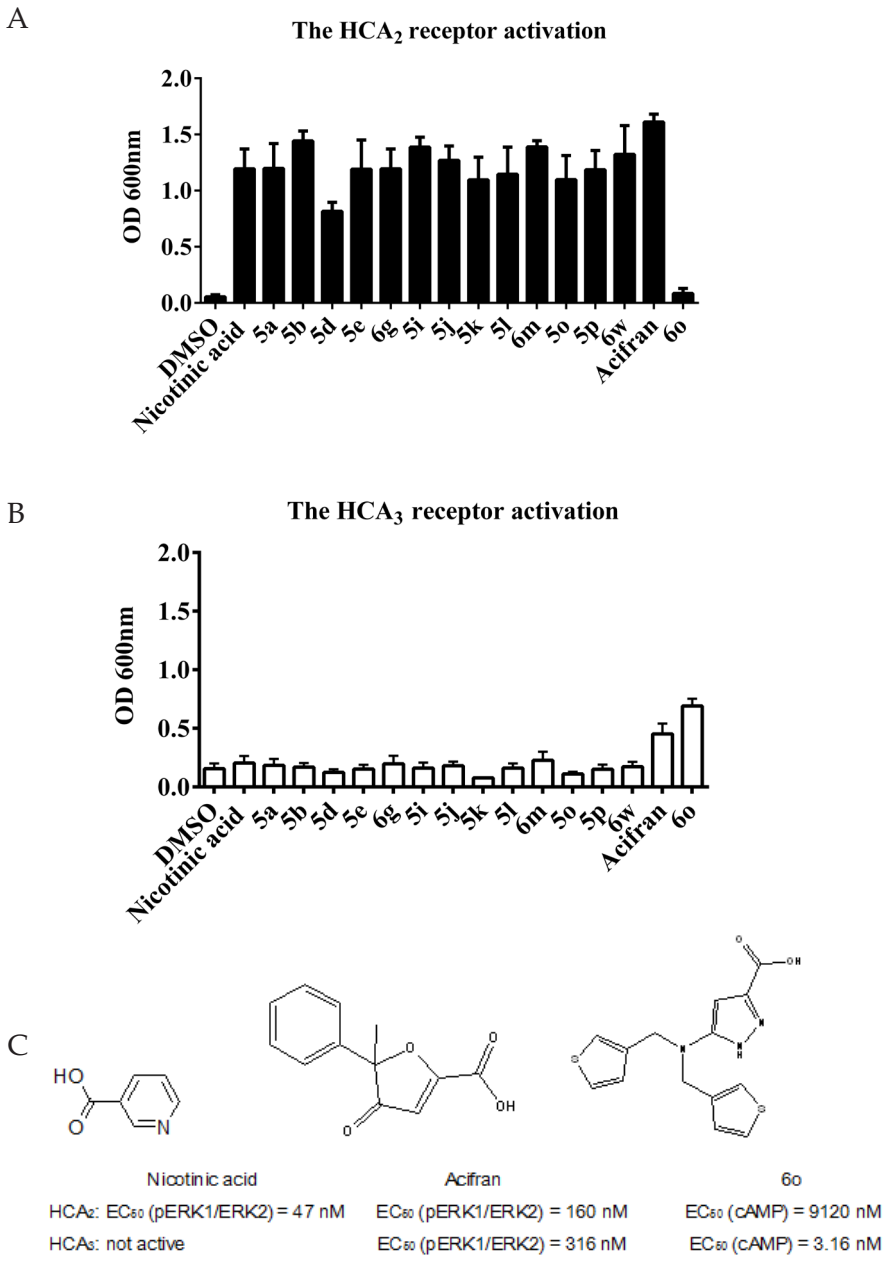

Fig. S1. HCA_2 receptor activation (A) and HCA_3 receptor activation (B) were tested in yeast liquid growth assays in the absence of ligand (DMSO) or in the presence of 100 μM of selected derivatives, or nicotinic acid, or acifran or reference ligand **6o**[1]; (C) Chemical structures of reference ligands: nicotinic acid[2], acifran[2], and **6o**[1].

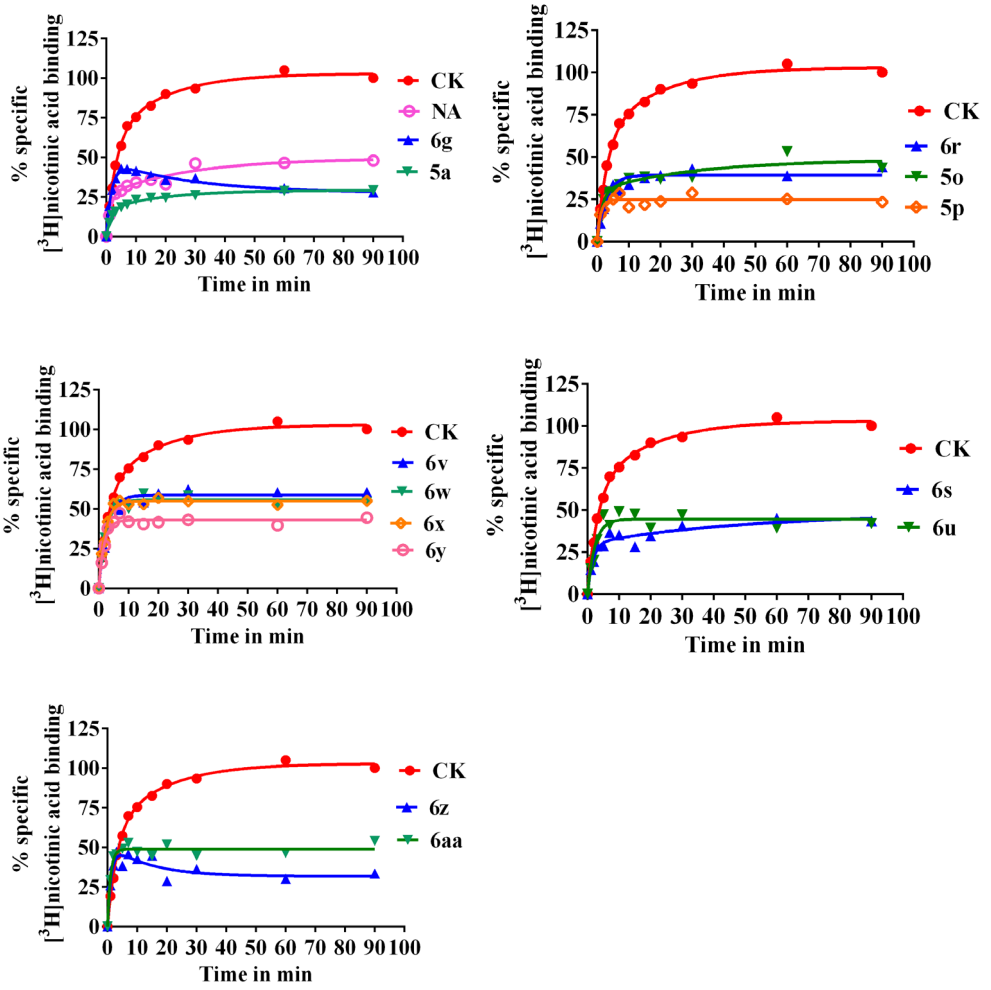

Fig. S2. [^{3}H]nicotinic acid competition association assay in the absence of ligand (control CK) and in the presence of 1 × IC_{50} of unlabeled ligands. Representative graphs from one experiment performed in duplicate. (A) **Nicotinic acid** (NA), **5a** (non-substituted) and **6g** (4-OH) yield their kinetic profiles in Table 1; (B) Mono-substituted analogues at 2-position: **6r** (2-OH), **5o** (2-Cl) and **5p** (2-F) yield their kinetic profiles in Table 1; (C) Di-substituted 2-R, 4-OH analogues: **6v** (2-Me), **6w** (2-Cl), **6x** (2-F) and **6y** (2-OH) yield their kinetic profiles in Table 2; (D) Di-substituted 3-R, 4-OH analogues: **6s** (3-Me), **6u** (3-F) yield their kinetic profiles in Table 2; (E) Tetrahydroanthranilic acid derivative **6z** and the thiophene bioisostere **6aa** yield their kinetic profiles in Table 3.

Selectivity assay

Transformation in a S. cerevisiae strain (MMY24)

The p426GPD_HCA_2 or p426GPD_HCA_3 plasmid was transformed according to the Lithium-Acetate procedure[3] into an engineered Saccharomyces Cerevisiae (S. cerevisiae) yeast strain, MMY24, expressing one specific Gpa1p/$G_{\alpha i3}$ chimeric G protein. The yeast strain was derived from the MMY11 strain and further adapted to communicate with mammalian GPCRs. Hereto the last five amino acid residues of the C-terminus of Gpa1p/$G_{\alpha i3}$ chimera had been replaced with the same-length sequence from mammalian $G_{\alpha i3}$ protein.[4, 5] The genotype of the MMY24 strain is: MATahis3 leu2 trp1ura3can1 gpa1_::G_i3 far1::ura3 sst2_::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZste2_::G418R and the sequence of these last 5 C-terminal amino acid residues of Gpa1p/$G_{\alpha i3}$ chimera is $ECGLY^{COOH}$[4, 5].

Liquid growth assay

The degree of receptor activation was measured by the growth rate of the yeast on histidine-deficient medium via the *FUS1-HIS3* reporter gene induction, which was described in our previous research[6] except that nicotinic acid was omitted from the YNB-UL mix (YNB + adenine + tryptophan + histidine, lacking uracil and leucine). Both the HCA_2 receptor and HCA_3 receptor were tested in liquid growth assays in the absence of any ligand (DMSO) or in the presence of 100 μM of selected derivatives, or nicotinic acid (Sigma, The Netherlands), or acifran (Tocris, USA) or reference ligand **6o**[1] (synthesized by Jacobus P. D. van Veldhoven again in our lab). Yeast cells with the HCA_2 receptor from an overnight culture were diluted to around 2×10^4 cells/mL ($OD_{600}\approx 0.001$) and 50 μL was added into each well (approx. 1,000 cells/well). Results were obtained from three independent experiments, performed in duplicate. Yeast cells with the HCA_3 receptor were diluted to approx. 2×10^5 cells/mL ($OD_{600} \approx 0.01$) and 50 μL was added into each well (approx. 1×10^4 cells/well). Results were obtained from two independent experiments, performed in duplicate.

Emax values of the liquid assay were assessed from the nonlinear regression package Prism 5.0 (GraphPad Software Inc., San Diego, CA).

References

[1] Skinner, P.J., et al. *5-N, N-Disubstituted 5-aminopyrazole-3-carboxylic acids are highly potent agonists of GPR109b.* Bioorg. Med. Chem. Lett. (2009) 19: 4207-4209.

[2] Mahboubi, K., et al. *Triglyceride modulation by acifran analogs: activity towards the niacin high and low affinity G protein-coupled receptors HM74A and HM74.* Biochem. Biophys. Res. Commun. (2006) 340: 482-490.

[3] Gietz, D., et al. *Improved method for high efficiency transformation of intact yeast cells.* Nucleic Acids Res. (1992) 20: 1425.

[4] Dowell, S.J. and Brown, A.J. *Yeast assays for G protein-coupled receptors.* G Protein-Coupled Receptors in Drug Discovery (2009) 552: 213-229.

[5] Dowell, S.J. *Yeast assays for G-protein–coupled receptors.* Receptors and Channels (2002) 8: 343-352.

[6] Liu, R., et al. *A yeast screening method to decipher the interaction between the adenosine A_{2B} receptor and the C-terminus of different G protein α-subunits.* Purinergic Signal. (2014) 10: 441-453.