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Allosteric Functional Switch of Neurokinin A-mediated Signalling at the Neurokinin NK2 Receptor: Structural Exploration

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ABBREVIATIONS LIST FOOTNOTE. ^a Abbreviations: FRET, fluorescence resonance energy transfer;
BRET, bioluminescence resonance energy transfer; GPCR, G-protein-coupled receptor; EGFP,
enhanced green fluorescent protein; NK2R, NK2 receptor; NKA-Bo, NKA linked to the Bodipy
fluorophore; NK2-EGFP, NK2 receptor expressed as a fusion protein with the enhanced green
fluorescent protein on the N-terminal;

1 ABSTRACT. The neurokinin NK2 receptor is known to pre-exist in equilibrium between at least three
2 states: resting-inactive, calcium-triggering and cAMP-producing. Its endogeneous ligand, NKA, mainly
3 induces the calcium response. Using a FRET^a-based assay, we have previously discovered an allosteric
4 modulator of the NK2 receptor that has the unique ability to discriminate among the two signaling
5 pathways: Calcium-signaling is not affected while cAMP signaling is significantly decreased. A series
6 of compounds have been prepared and studied in order to better understand the structural determinants
7 of this allosteric functional switch of a GPCR. Most of them display the same allosteric profile, with
8 smooth pharmacomodulation. One compound however exhibits significantly improved modulatory
9 properties of NKA induced signaling when compared to the original modulator.

10 KEYWORDS. Allosteric ligands; FRET; NK2 neurokinin receptor, functional switch, structure-activity
11 relationship.

12

13 MANUSCRIPT TEXT.

14 **Introduction.** Allosterism is a well known process that has been extensively studied in enzymes and
15 in some receptors. However, the study of this phenomenon for G protein-coupled receptors (GPCR^a) is
16 relatively recent, probably due to the lack of simple detection methods.^{1,2} Since allosteric interactions
17 could be quite subtle, many ways of studying allosteric behaviors were established and can be used
18 individually or in tandem. Among them, the study of the effect of an allosteric ligand on the dissociation
19 of a labeled ligand to a receptor, which measures the ability of an allosteric ligand to alter the
20 dissociation rate constant (K_{off}) of the orthosteric labeled ligand in time, is one of the most sensitive,
21 direct and unequivocal method to validate an allosteric interaction.

22 We have demonstrated that fluorescence resonance energy transfer³ (FRET) represents an efficient
23 and informative way to study GPCR-ligand interaction.⁴ Indeed, a very specific and sensitive FRET
24 signal can be observed when an appropriate fluorescent ligand binds to a GPCR fused to a fluorescent

1 protein, such as enhanced green fluorescent protein (EGFP).⁵ Binding can be easily analyzed by a simple
2 measurement of EGFP emission. We have used FRET-based binding analysis to study several GPCRs⁶
3 including the neurokinin A (NKA) receptor, known as NK2 receptor. In this particular case, FRET
4 revealed that the recombinant human neurokinin NK2 receptor, in presence of NKA-Bo (NKA linked to
5 Bodipy^{4,7}), exists in equilibrium between predominantly three states: inactive (R), calcium-triggering
6 (conformation A1) characterized by a rapid association and dissociation of the endogeneous agonist
7 NKA, and cAMP-producing (conformation A2), characterized by slow association and slow dissociation
8 of the agonist.

9 We have screened 1,600 drug-like molecules issued from our laboratory in a FRET-based binding
10 assay in order to find antagonist ligands. Interestingly, this study led to the discovery of compound **1**
11 (Chart 1), a new allosteric ligand for the neurokinin NK2 receptor. This ligand exhibited the particularity
12 of being a strong negative allosteric modulator for the cAMP-producing conformation (A2) while not
13 affecting or slightly potentiating the calcium triggering conformation (A1).⁸ In fact, in the presence of 20
14 nM of NKA-Bo, the receptor was stabilized in a 30/70 ratio of A1/A2 conformations. Upon addition of
15 10 μ M of **1**, still in presence of 20 nM of NKA-Bo, the NK2 receptor was stabilized in a 50/50 ratio of
16 A1/A2 conformations. Finally, addition of 50 μ M of **1** balances the receptor to a 95/5 maximal ratio of
17 A1/A2 conformations (Table 1).⁸

18 We report here the structure-activity relationship study around this allosteric functional switch of a
19 GPCR, the neurokinin NK2 receptor, and characterize our best optimized hit, compound **16**, in both
20 calcium mobilization and cAMP accumulation assays.

21

22 **Methods**

23 **Chemistry.** The synthesis of **1**, [N-benzyl,N-(2-naphthylmethyl)-amino]-acetonitrile (Chart 1), has
24 previously been reported.^{8,9} Synthesis of 31 derivatives showing structural similarity to **1** was
25 undertaken, based on the substitution of the three groups around the central tertiary amine. The 32
26 compounds were synthesized according to standard procedures (Scheme 1). Two methods were

1 followed to obtain the whole set of compounds. Method A allowed the synthesis of 22 compounds (**1-**
2 **20; 31, 32**) by two successive steps, a reductive amination between an aldehyde and an amine exhibiting
3 the R2 group followed by an alkylation of the secondary amine. Further 8 compounds were synthesized
4 by converting the N,N-(2-methylnaphthyl-benzyl)-2-aminoethanoic acid **18** into bulky amides **25-28** or
5 bulky esters **21-24** in peptide coupling conditions. Method B was followed to synthesize two additional
6 compounds, by condensing first an aldehyde and an amine exhibiting the R2 group, followed by an
7 alkylation of the secondary amine, leading to the compounds **29** and **30**. Yields and chemical, physical
8 and spectroscopic characteristics of **1** and its 31 analogues are reported in the Experimental section and
9 in Supporting Information.

10
11 **Biology.** The FRET-based assay was a measurement of fluorescence emission of EGFP as a function
12 of time at 510 nm.^{4,6-8} It proceeds through three different phases (Figure 1) and allows to determine if a
13 non fluorescent molecule behaves as a competitor of the fluorescent molecule or if it rather modulates
14 the interaction of the fluorescent molecule with the receptor. The phase P1 corresponds to the real time
15 recording of the association of fluorescent NKA-Bo (20 nM) to EGFP-labelled NK2 receptor. The
16 observed decrease of EGFP fluorescence emission at 510 nm reflects energy transfer from excited EGFP
17 (linked to the receptor) to the fluorophore bodipy (linked to neurokinin A) that develops over time. The
18 phase P2 corresponds to the incubation period with the compound (10 μ M) to be tested. If the test
19 compound is a competitor of NKA-Bo, an upward deflection is detected. This reflects partial-to-total
20 dissociation of NKA-Bo from its binding site and consequent reduction of energy transfer. Alternatively,
21 the compound may either not bind to the receptor or may behave as an allosteric modulator. In this case
22 no signal would be detected during phase P2. The final phase, P3, corresponds to NKA-Bo dissociation
23 from NK2-EGFP receptor following addition of 20 μ M of unlabeled NKA. During this phase, each
24 NKA-Bo molecule that dissociates from its binding site is replaced by an unlabelled NKA molecule
25 present in large excess. The rate of NKA dissociation, determined from the fitting of phase P3

dissociation trace, is thus directly linked to the dissociation constant of NKA-Bo for a given conformation of the NK2 receptor. In previous studies,^{6,8} fluorescent NKA was shown to dissociate from two conformations of the NK2 receptor, the relative abundance of which depended on the incubation time or on the pharmacological agent added to the preparation. A rapid dissociation rate associated to a low affinity conformation characterized as a calcium signaling state was identified, together with a slow dissociation rate corresponding to a high affinity cAMP-signaling state. In the present work, dissociation traces are fitted using this two state model with the equation: $F(t) = \lambda_1 (k_1 \times t) + \lambda_2 (k_2 \times t)$, where $F(t)$ is the variation of fluorescence over time, λ_1 and λ_2 were the amplitudes of the rapid and slow dissociation processes characterized by rate constants k_1 and k_2 , respectively.

Best fits of dissociation traces were obtained according to Maillet et al.⁸ with two exponential relaxations. The relative amplitudes λ_1 and λ_2 for rapid and slow dissociations were fitted using the least square method and assigned to the corresponding conformations denominated A1 (for rapidly dissociating, calcium signaling) and A2 (for slowly dissociating, cAMP-signaling), depending on the compound present. When test compounds did not exhibit a competitive character (all but compounds **11**, **30**, **31**, **32**) determination of relative A1 and A2 abundances did not require rapid and slow rate constants to be fixed. For compounds **11**, **30**, **31**, **32**, the residual amplitude of dissociation in the presence of NKA was reduced and rate constant determinations were less precise. For the sake of homogeneity, all data are presented here with imposed rate constants equal to 0.03 sec⁻¹ for rapid dissociation, and 0.006 sec⁻¹ for slow dissociation. When excess of known competitive ligands such as unlabeled NKA or the competitive NK2 receptor antagonist SR 48968 were used as competitors, dissociation of NKA-Bo followed a bi-exponential time course with a rapid dissociation rate ($k_1 = 0.03$ sec⁻¹) accounting for 70% of the signal and a slow dissociation rate ($k_2 = 0.006$ sec⁻¹) accounting for 30% of the signal amplitude, resulting in a 30/70 ratio A1/A2 conformations as described in Maillet et al.⁸

1 **Results and discussion.** The FRET-based screening at 10 μ M concentration of 1,600 compounds
2 from our laboratory collection on the NK2-EGFP receptor led to the discovery of compound **1** (Chart 1).
3 This hit was unable to fully reverse the binding of NKA-Bo alone but in the presence of an excess of
4 NKA, compound **1** significantly accelerated the dissociation kinetics of NKA-Bo (Table 1), showed no
5 effect on (or weak potentiation of) the calcium-induced pathway (Figure 2) and dramatically inhibited
6 NKA-induced cAMP response of the receptor (Figure 3). Because the dissociation rate of an orthosteric
7 ligand can only be modified by a ligand able to bind simultaneously to the receptor, and therefore, on a
8 topographically different binding site than the orthosteric ligand, the phenomenon of allosteric
9 interaction between compound **1** with NKA-Bo to the neurokinin NK2 receptor was validated and
10 confirms the existence of a <NKA-compound **1**-NK2 receptor> ternary complex. Analysis of association
11 kinetics of NKA revealed that **1** does not alter the affinity of the agonist for the different receptor
12 conformations but rather prevents the formation of the 'cAMP-triggering conformation' while not
13 affecting the formation of 'calcium-triggering conformation' of the NKA-NK2R complex.⁸ Compound **1**
14 was thus identified as the first allosteric modulator of the neurokinin 2 receptor that discriminates
15 among biologically active conformations of the receptor and exerts a selective, allosteric functional
16 switch of a GPCR upon endogenous ligand activation. Our aim here was to explore structural elements
17 of the structure of **1** that determine its potential to increase the population of A1 conformation at a 10
18 μ M concentration of allosteric ligand.

19 We attempted to modify the structure of **1** to obtain analogs with as low as possible competitive
20 character and as high as possible modulating properties. Compounds are validated, using the FRET-
21 based assay, by i) a lack of inhibition of NKA-Bo binding following the addition of the test compound
22 and ii) an increase in the fraction of receptors inducing calcium production, and further characterized in
23 functional assays. In this study, we report the structure and the allosteric activity of 31 analogues of **1**.
24 Their ability to induce an inhibition of FRET by themselves or to affect NKA-Bo dissociation kinetics at
25 10 μ M was analyzed. Most compounds interact with the NK2R. With the exception of compounds **5**, **11**,
26 **30**, **31** and **32** which could not be assigned as competitive agents or as negative allosteric effectors of

NKA-Bo binding, all other ligands behave as non competitive, allosteric switches favoring the NKA-Bo induced calcium signaling states at the expense of the NKA-induced cAMP signaling (Tables 2, 3 and 4). We first modified the benzyl substituent (R1) of **1** (Chart 1 and Table 2). An introduction of a halogen atom on the benzyl ring either in position 4 or 2, such as in compounds **2**, **3**, and **4**, lead to a non significant reduction in the allosteric activity of the ligands (3-5% reduction of A1 conformation). Another modification spacing the aromatic ring from the central nitrogen atom such as replacement of the benzyl by a phenethyl group in analog **5**, did not improve the efficacy of the allosteric ligand. In contrast, introduction of an electron donor group, such as a 4-methoxy-benzyl in **6**, seemed to lead to a slightly better efficacy (54% of A1 conformation). If the substitution of the benzyl domain by halogen atoms had a slightly negative effect on the allosteric activity of the compounds, substitution with an electron donor group weakly reinforced the allosteric activity, but overall, the substitution of the benzyl group did not have a significant impact on the modulation of allosteric activity.

The importance of the naphthalen-2-ylmethyl moiety (Ar) of molecule **1** was then explored (Table 3). Its replacement by halogeno-benzyl groups, such as in compounds **7** and **8**, had little influence on the allosteric activity (44 to 53 % of A1 conformation). Replacement with an indol (analogue **9**) resulted in a cytotoxic compound which could not be studied further. The quinolinyl group in **10** and **11**, which could be viewed as hydrogen bond acceptor, did not lead to compounds better than **1**. However, it is noteworthy that among the quinolinyl analogues, the quinolin-3-ylmethyl one (**11**) promoted a strong inhibition of FRET, supporting a competitive binding in addition to allosteric modulation, while the quinolin-2-ylmethyl one (**10**) conserved a pure non-competitive allosteric behavior.

In order to probe the importance of the rigidity of the bicycle in **1**, the cinnamyl analogue **12** was synthesized. This compound exhibited an allosteric activity quite similar to **1** (47% of A1 conformation). Interestingly, replacement of the naphthalen-2-ylmethyl moiety with a benzyl group substituted by an electron donating –OMe (**13**) or an electron withdrawing –CF₃ group (**14**) had no effect on the activity of the ligands. Taken together, these results indicate that replacing the naphthalen-2-ylmethyl group by diverse aromatic moieties did not increase significantly the allosteric modulator

activity. This suggests that the Ar group located near the tertiary amine does not make strong specific interactions with the receptor and is most probably involved in the establishment of aromatic-aromatic interactions between the allosteric ligand and the allosteric site of the receptor.

The contribution of the acetonitrile substituent (R2) of the central nitrogen was then investigated (Table 4). The carbonitrile was first replaced by an alkyne group (compound **15**). This compound showed an allosteric activity corresponding to 51% A1 conformation, thus equally active to compound **1**, suggesting that the nitrile was not involved in any hydrogen bond but rather acted as an electron rich, polarisable moiety. The location of the nitrile was then explored in preparing compound **16** which is the superior homolog of **1**. Interestingly, the allosteric activity was significantly improved up to 68% A1 conformation, suggesting the creation or the re-enforcement of an interaction between the nitrile function and the allosteric site. To further explore the carbonitrile modifications, an ester or an acid function was introduced in **17** or **18**, respectively. The ethyl ester **17** retained a good allosteric activity (51% of A1 conformation), as efficient as **1**, without promoting an inhibition of FRET. In contrast, the corresponding acid derivative **18** lost some of its allosteric activity with promoting only 35% of A1 conformation. The elongation of the chain to four methylene groups (compounds **19** and **20**) afforded an equi-active ester and an acid with some rescued allosteric activity (52% and 44% of A1 conformation, respectively). The influence of the ester was then further explored with some derivatives such as compounds **21** to **24**. Alkyl or aromatic hydrophobic esters **21** and **22** showed decreased activity. A short hydrophilic hydroxyethyl chain provided compound **23** as active as **1**. The more bulky and basic chain, as found in **24**, slightly decreased the allosteric activity to 42% of A1 conformation. Amides were then logically synthesized and tested. Four compounds, **25** to **28** were prepared. The N-butylamide **25** was as active as **1** but the cyclic piperidino analogue **26** was significantly less active than **1**, with 44% of A1 conformation. The N'-methylpiperazin,N-acetamide **27** was more active than **1** promoting 58% of A1 conformation, while the N'-phenyl derivative **28** was much less active than **1**, suggesting a positive contribution of the additional N' basic atom, possibly related to its basicity.

In a final round of chemical modifications, we replaced the carbonitrile function with electron-rich heteroaromatic groups. Compounds **29** to **32** were synthesized. They all exhibited an allosteric modulator activity superior to **1**, but most of them (**30-32**) developed a significant inhibition of FRET intensity, supporting the notion that some competition with NKA may also be occurring.

Taken all together, the results obtained in the present study correspond to an improvement in term of allosteric activity supporting the calcium-induced pathway. However, the structure-activity relationship appears difficult to analyze since the allosteric activity remained very mildly sensitive to very diverse structural modifications. This led us to suggest that the interaction with the binding site might not involve very strong, **oriented and specific bonds such as H-bonds** but rather a set of diffuse hydrophobic, aromatic interactions with the allosteric site of the NK2 receptor. In order to find a possible explanation for these results we examined the three-dimensional model of the NK2 receptor derived from the bovine opsin crystal structure.¹⁰ To be consistent with our structure-activity relationship data we looked for aromatic domains accessible to ligands. Only two domains could clearly be identified (Figure 4). The first one was located at the entrance of the binding cleft, at the top of transmembrane domains TM1 and TM2 which corresponds to the cluster of three aromatic amino acids, Trp37(1.35), Tyr41(1.39) and Tyr93(2.64) (in green in the left panel, Figure 4). This pocket could be located in the direct neighborhood of the agonist binding site,^{11,12} which could account for an inhibition of FRET by exerting competitive interaction with the orthosteric ligand and could support the hypothesis of a competitive component of our ligands. Interestingly, a second aromatic cluster has been found at the top of transmembrane helices TM2 and TM3, pointing towards the *outside* of the helix bundle, that is toward the membrane (in blue in the right-hand panel, Figure 4). It encompassed five phenylalanine residues namely Phe85(2.56), 89(2.60), 105(3.24), 108(3.27) and 114(3.31). Such a cluster is unusual in GPCRs and could be on one hand, the binding site of compound **1**, whereby promoting a conformational change in the receptor resulting in an increase of the calcium-induced pathway. Alternatively, it is now widely accepted that GPCRs exist and might function as dimers and allosteric ligands might modulate the homo

1 or heterodimerization of GPCRs.¹³ A great number of GPCRs were shown to form homodimers or
2 multimers by Western blotting, by co-immuno-precipitation in transfected/infected cells or, more
3 recently, by “resonance energy transfer” techniques in transfected living cells (fluorescence and
4 bioluminescence resonance energy transfer: (FRET or BRET)).¹⁴ Therefore, we suggest that these
5 phenylalanine residues could represent an attachment point between the protomers. The same cluster can
6 be found in homology models of NK2-R derived from the most recently published crystallographic
7 structures of the adrenergic and adenosine receptors.¹⁵⁻¹⁷ Thus, one may hypothesize that compound **1**
8 may bind this aromatic bud and thereby either reinforce or disrupt the ability of protomers to dimerize,
9 resulting in conformational and functional switches.¹⁸ Though still speculative, the atypical features and
10 location of this aromatic cluster is worth mentioning to trigger experimental evaluation of its functional
11 relevance.

12 To summarize, only the modifications of the carbonitrile function of compound **1** were able to
13 significantly increase the allosteric effect of the ligands, leading to an optimised hit, compound **16**.
14 Therefore, to further characterize the ability of compound **16** to increase the population of A1
15 conformation, intracellular calcium mobilization assays as well as cAMP accumulation were
16 investigated at the NK2 receptor. Figure 2 shows that **1**, as previously reported,⁸ did not modify the
17 intracellular calcium response to a significant extent ($EC_{50} = 0.55 \pm 0.05$ nM for NKA alone and $EC_{50} =$
18 0.50 ± 0.05 nM for NKA in presence of 20 μ M of **1**). We note in this work that the EC_{50} values of NKA
19 for calcium signaling, as well as for cAMP-signaling, are approximately 10-fold lower than those
20 obtained previously.⁸ Consequently, inhibition of cAMP-responses by compound **1** looks less efficient.
21 These changes are accounted for by inter-batches variations in surface expression of the EGFP-NK2
22 receptor as demonstrated by other groups.^{19,20} The activity of all compounds studied in the present work
23 was evaluated in a newly selected cell line expressing EGFP-NK2R. Interestingly, compound **16** did not
24 promote any drastic change in NKA potency ($EC_{50} = 0.62 \pm 0.04$ nM), but exhibited a significant
25 increase of the agonist efficacy ($E_{max} = 120 \pm 5\%$), which reached a plateau approximately 20% higher
26 than in control conditions, confirming that the butyronitrile group was definitely able to improve the

biological activity of the modulator. Even more interestingly, in a cAMP accumulation assay (Figure 3), compound **16** appeared more potent than compound **1**, such that while compound **1** (as previously reported⁸) was able to reduce the maximal effect of NKA of 33%, compound **16** at an identical concentration, reduced the agonist efficacy of almost 50%. These results suggest that, if compound **1** was exerting its allosteric effect unilaterally by only inhibiting the cAMP-induced response of NKA at the NK2 receptors while having no significant effect on the calcium response of the endogenous agonist, compound **16** is actually able to, on one hand potentiate the efficacy of NKA in calcium mobilization assay, while on the other hand, inhibit the efficacy of the endogenous agonist-induced cAMP response.

Allosteric modulation of GPCRs is now a well established concept,^{1,2,13,18,21,22,23} originally defined by the International Union of Pharmacology (IUPHAR: <http://www.iuphar.org/>) committee as ‘modulators that enhance the affinity and/or efficacy of the orthosteric ligand while having no effect on their own’.²⁴ Indeed many modulators that enhance or diminish the effects of agonists or antagonists on a variety of GPCRs are described, some of them reaching the clinical phase.²¹ On another hand, molecules have been described as being able to trigger on their own different signaling pathways upon binding to a given GPCR.²⁵ This mechanism has been referred to as ‘agonist-’ or ‘ligand-directed trafficking of receptor stimulus’.²⁵⁻²⁷ Compounds **1** and **16** open new perspectives since they combine both effects, allostery and stimulus trafficking: they have no effect on their own but they are able to traffic the stimulus of the endogenous ligand, NKA, from the cAMP to the Calcium signaling pathway. They are thus pure allosteric functional switches of the endogenous ligand. Such a pharmacological profile had been reported in 2006 for an ionic species, gadolinium (Gd^{3+}), acting at the class III metabotropic glutamate receptor mGluR_{1a}.²⁸ To our knowledge, compound **1** was the first drug-like, allosteric switch of the function triggered by the endogenous ligand of a Class I GPCR.⁸ A similar profile has been very recently considered for benzoylthiophenes modulating the function of a non-endogenous agonist of the A1 adenosine receptor.²⁹ Compound **16** represents a relatively more potent allosteric modulator than **1** in the studied system. Its ability to switch the function of the endogenous ligand NKA in other cell types and in tissues relevant to human pathologies will have to be explored. However, the present results

1 further illustrate the possibility to discover such molecules and pave the way for not only receptor-
2 selective but also signaling-pathway selective therapies under the physiological control of the
3 endogenous ligand release.

4
5 **Conclusion.** We have synthesized more than thirty compounds based on compound **1**, an allosteric
6 functional switch of a class I GPCR. Using a FRET based assay, we have characterized most of them as
7 allosteric modulators of the NK2 receptor. These allosteric modulators exhibited the particularity of
8 stabilizing the receptor in the calcium-triggering state (Gq coupling) to the detriment of the cAMP
9 synthesizing state (Gs coupling). Our structure-activity relationship study led to the optimization of its
10 allosteric activity, especially with the analogue **16** which enhanced the percentage of A1 conformation
11 of NK2 receptor to 68% at 10 μ M and increased the function switch amplitude. We have now a new
12 efficient allosteric modulator that can target specifically one among two signaling pathways activated by
13 the endogenous ligand at a given receptor. This thus opens the road to a superior level of specificity for
14 ligands acting at GPCRs, but their detailed mechanism of action as well as their functional and
15 therapeutic potential remains to be explored.

16 17 **Experimental section**

18 **Chemistry.**

19 **General.** All chemicals used were of reagent grade. All commercialized compounds were purchased
20 from Accros, Aldrich, Avocado, Fluka, Lancaster, and used without further purification. Progress of the
21 reactions was monitored by TLC on silica gel plates. Extracts were dried over MgSO₄, and solvents
22 were removed under reduced pressure. Merck silica gel (Kieselgel 60) was used for flash
23 chromatography (230-400 mesh) columns. Melting points were determined using a Mettler FP62
24 apparatus and are uncorrected. ¹H NMR and ¹³C NMR spectra were recorded on Bruker DPX 200 MHz
25 and 300 MHz spectrometers with TMS as internal standard; the values of the chemical shifts are given
26 in ppm. Mass spectra were determined with a Perkin Elmer ESI-TOF apparatus (dilution of the product

1 to a concentration of 10^{-6} in MeOH/H₂O=90/10). LC/MS spectra were obtained on a ZQ (Z quadripole)
2 Waters/Micromass spectrometer equipped with an X-Terra C18 column (4.6 x 50 mm, 3.5 μ m) using
3 electrospray ionisation mode (ESI) with a linear gradient from water (0.1% TFA) to CH₃CN (0.1%
4 TFA). Retention times (Rt) from analytical RP-HPLC are reported in minutes. HRMS spectra were
5 obtained on a MicroTof mass spectrometer from Bruker using electrospray ionisation (ESI) mode and a
6 time-of-flight analyser (TOF). Elemental analyses were performed on a Perkin-Elmer 240C elemental
7 analyzer, and the results are within $\pm 0.4\%$ of the theoretical values. Yields refer to purified products and
8 are not optimized. Purity of tested compounds was superior to 95% as ascertained by elementary
9 analysis or LC-MS (see Experimental section and Supporting Information). The identity of the tested
10 compounds was ascertained by ¹H NMR, ¹³C NMR and LC/MS (see Experimental section and Supporting
11 Information).

12 **General procedure for the synthesis of secondary amines.** A mixture of aldehyde (1.0 eq) and
13 substituted benzylamine (1.0 eq) in MeOH (0.3 M) was first heated at 60 °C during 4 h and cooled at 0
14 °C, NaBH₄ (3 eq) was added to the reaction mixture and the solution was stirred for 4 h at RT. After
15 removal of the solvent, the solid residue obtained was dissolved in AcOEt. The organic layer was
16 washed with a saturated solution of NaHCO₃ (x2), water, saturated solution of NaCl, then dried and
17 reduced to yield an oil (40-99%). The oil was used without further purification.

18 **General procedure for the synthesis of tertiary amines.** To a mixture of secondary amine (1.0 eq)
19 and TEA (1.2 eq) in DMF (0.3 M) was added dropwise a solution of halogenated compound (1.1 eq) in
20 DMF (0.3 M). The solution was stirred overnight at 60°C and the solvent was removed in vacuo. The
21 resulting oil was dissolved in AcOEt; the organic layer was washed with a saturated solution of
22 NaHCO₃ (x2), water, saturated solution of NaCl, then dried and reduced. The resulting product was
23 purified by flash chromatography to yield an oil (32-98%).

24 **General procedure for the synthesis of amides.** To a mixture of N,N-(2-naphthylmethyl-benzyl)-2-
25 aminoethanoic acid (1.0 eq) and N-methylmorpholine (3.2 eq) in DMF (0.3 M) was added a solution of
26 substituted amine (1.1 eq) in DMF (0.3 M) followed by the coupling agent BOP. The solution was

1 stirred for 1 night at RT and reduced. The resulting oil was dissolved in AcOEt; the organic layer was
2 washed with water, with a saturated solution of NaCl, dried and the solvent was removed in vacuo. The
3 resulting product was purified by flash chromatography to yield the expected product (67-96%).

4 **General procedure for the synthesis of esters.** To a mixture of N,N-(2-naphthylmethyl-benzyl)-2-
5 aminoethanoic acid (1.0 eq) and N-methylmorpholine (3.2 eq) in DMF (0.3M) was added a solution of
6 substituted alcohol (1.1 eq) in DMF (0.3 M) followed by the coupling agent BOP. The solution was
7 stirred overnight at RT and reduced. The resulting oil was dissolved in AcOEt; the organic layer was
8 washed with water, a saturated solution of NaCl, then dried and the solvent was removed in vacuo. The
9 resulting product was purified by flash chromatography to yield the expected product (35-84%).

10
11 **General procedure for the synthesis of salts.** HCl gas was bubbled for 2 minutes into a solution of
12 basic compound (0.3 M) in Et₂O or AcOEt. After evaporation the resulting product was collected as an
13 uncoloured powder.

14 **(2-Naphthylmethyl)-benzylamine (a).** Prepared from 2-naphtaldehyde (10 g, 64 mmol), benzylamine
15 (6.86 g, 64 mmol) and NaBH₄ (7.3 g, 196 mmol) using the general procedure for the synthesis of
16 secondary amines (14.4 g, 91%). R_f = 0.5 (Hexane/AcOEt=2/1). ¹H NMR (200 MHz, CDCl₃): δ 7.89-
17 7.82 (4H, m, H arom.), 7.55-7.28 (8H, m, H arom.), 4.02 (2H, s, -CH₂-napht.), 3.89 (2H, s, -CH₂-benz.).

18 **2-Naphthylmethyl)-4-bromobenzylamine (b).** Prepared from 2-naphtaldehyde (2.34 g, 15 mmol), 4-
19 bromobenzylamine (2.79 g, 15 mmol) and NaBH₄ (1.7 g, 45 mmol) using the general procedure for the
20 synthesis of secondary amines (3.42 g, 70%). R_f = 0.3 (Hexane/AcOEt=9/1). ¹H NMR (200 MHz,
21 CDCl₃): δ 7.85-7.81 (4H, m, H arom.), 7.49-7.44 (3H, m, H arom.), 7.29-7.28 (4H, m, H arom.), 3.96
22 (2H, s, -CH₂-napht.), 3.80 (2H, s, -CH₂-bromobenz.).

23 **(2-Naphthylmethyl)-2-bromobenzylamine (c).** Prepared from 2-naphtaldehyde (1.4 g, 9 mmol), 2-
24 bromobenzylamine (1.67 g, 9 mmol) and NaBH₄ (1 g, 27 mmol) using the general procedure for the
25 synthesis of secondary amines (2.55 g, 87%). R_f = 0.5 (Hexane/AcOEt=2/1). ¹H NMR (200 MHz,

1 CDCl₃): δ 7.89-7.85 (4H, m, H arom.), 7.53-7.34 (5H, m, H arom.), 7.33-7.31 (1H, m, H arom.), 7.22-
2 7.19 (1H, m, H arom.), 4.02 (2H, s, -CH₂-napht.), 3.98 (2H, s, -CH₂-benz.).

3 **(2-Naphthylmethyl)-4-iodobenzylamine (d)**. Prepared from 2-naphtaldehyde (2 g, 12.8 mmol), 4-
4 iodobenzylamine (2.98 g, 12.8 mmol) and NaBH₄ (1.5 g, 38.4 mmol) using the general procedure for the
5 synthesis of secondary amines (4.58 g, 96%). R_f = 0.25 (Hexane/AcOEt=9/1). ¹H NMR (200 MHz,
6 CDCl₃): δ 7.85-7.81 (5H, m, H arom.), 7.49-7.44 (4H, m, H arom.), 7.29-7.28 (2H, m, H arom.), 3.96
7 (2H, s, -CH₂-napht.), 3.80 (2H, s, -CH₂-iodobenz.).

8 **(2-Naphthylmethyl)-4-phenethylbenzylamine (e)**. Prepared from 2-naphtaldehyde (73 mg, 0.46
9 mmol), 4-phenethylbenzylamine (100 mg, 0.46 mmol) and NaBH₄ (50 mg, 1.38 mmol) using the
10 general procedure for the synthesis of secondary amines (250 mg, 99%). R_f = 0.25
11 (Hexane/AcOEt=8/2). ¹H NMR (200 MHz, CDCl₃): δ 7.80-7.78 (4H, m, H arom.), 7.51-7.46 (3H, m, H
12 arom.), 7.30-7.18 (9H, m, H arom.), 3.98 (2H, s, -CH₂-napht.), 3.83 (2H, s, -CH₂-benz.), 2.93 (4H, s, -
13 CH₂-CH₂-).

14 **(2-Naphthylmethyl)-4-methoxybenzylamine (f)**. Prepared from 2-naphtaldehyde (500 mg, 3.2
15 mmol), 4-methoxybenzylamine (427 mg, 3.2 mmol) and NaBH₄ (365 mg, 9.6 mmol) using the general
16 procedure for the synthesis of secondary amines (736 mg, 83%). R_f = 0.35 (Hexane/AcOEt=9/1). ¹H
17 NMR (200 MHz, CDCl₃): δ 7.90-7.78 (4H, m, H arom.), 7.50-7.45 (3H, m, H arom.), 7.32-7.26 (2H, m,
18 H arom.), 6.93-6.88 (2H, m, H arom.), 3.98 (2H, s, -CH₂-napht.), 3.83 (5H, s, -CH₂-methoxybenz.).

19 **(3-Chlorobenzyl)-benzylamine (g)**. Prepared from 3-chlorobenzaldehyde (1 g, 7.1 mmol),
20 benzylamine (760 mg, 7.1 mmol) and NaBH₄ (810 mg, 21.3 mmol) using the general procedure for the
21 synthesis of secondary amines (1.48 g, 90%). R_f = 0.25 (Hexane/AcOEt=9/1). ¹H NMR (300 MHz,
22 CDCl₃): δ 7.78-7.72 (4H, m, H arom.), 7.49-7.43 (5H, m, H arom.), 4.32 (2H, s, -CH₂-chlorobenz.),
23 4.29 (2H, s, -CH₂-benz.).

24 **(4-Chlorobenzyl)-benzylamine (h)**. Prepared from 4-chlorobenzaldehyde (703 mg, 5 mmol),
25 benzylamine (535 mg, 5 mmol) and NaBH₄ (570 mg, 15 mmol) using the general procedure for the

1 synthesis of secondary amines (1.48 g, 90%). Rf = 0.45 (Hexane/AcOEt=7/3). ¹H NMR (200 MHz,
2 CDCl₃): δ 7.36-7.33 (4H, m, H arom.); 7.32-7.30 (5H, m, H arom.); 3.81 (2H, s, -CH₂-chlorobenz.);
3 3.79 (2H, s, -CH₂-benz.).

4 **N-(1H-Indol-3-ylmethyl)-N-benzylamine (i).** Prepared from indol-3-carboxaldehyde (725 mg, 5
5 mmol), benzylamine (540 mg, 5 mmol) and NaBH₄ (570 mg, 15 mmol) using the general procedure for
6 the synthesis of secondary amines (0.62 g, 52%). Rf = 0.85 (AcOEt). ¹H NMR (300 MHz, CDCl₃): δ
7 7.91-7.72 (4H, m, H arom.), 7.61-7.49 (4H, m, H arom.), 7.33 (1H, t, J = 7.2 Hz, H arom.), 7.20 (1H, t, J
8 =7.2 Hz, H arom.), 4.01 (2H, s, -CH₂-indol.), 3.98 (2H, s, -CH₂-benz.).

9 **N-(quinol-2-ylmethyl)-N-benzylamine (j).** Prepared from quinolin-2-carboxaldehyde (500 mg, 3.2
10 mmol), benzylamine (340 mg, 3.2 mmol) and NaBH₄ (365 mg, 9.6 mmol) using the general procedure
11 for the synthesis of secondary amines (0.64 g, 80%). Rf = 0.25 (Hexane/AcOEt=5/5). ¹H NMR (300
12 MHz, CDCl₃): δ 7.86-7.79 (2H, m, H arom.), 7.74-7.73 (1H, d, H arom.), 7.65-7.60 (1H, m, H arom.),
13 7.45-7.30 (7H, m, H arom.), 4.10 (2H, s, -CH₂-quinol.), 3.85 (2H, s, -CH₂-benz.).

14 **N-(quinol-3-ylmethyl)-N-benzylamine (k).** Prepared from quinolin-3-carboxaldehyde (500 mg, 3.2
15 mmol), benzylamine (340 mg, 3.2 mmol) and NaBH₄ (365 mg, 9.6 mmol) using the general procedure
16 for the synthesis of secondary amine (0.75 g, 95%). Rf = 0.25 (Hexane/AcOEt=5/5). ¹H NMR (300
17 MHz, CDCl₃): δ 8.90 (1H, s, H arom.), 8.12-8.08 (2H, m, H arom.), 7.81-7.79 (1H, m, H arom.), 7.79-
18 7.77 (1H, m, H arom.), 7.71-7.69 (1H, m, H arom.), 7.37-7.27 (5H, m, H arom.), 4.00 (2H, s, -CH₂-
19 quinol.), 3.87 (2H, s, -CH₂-benz.).

20 **N-(4-Trifluoromethylbenzyl)-benzylamine (l).** Prepared from 4-trifluorobenzaldehyde (500 mg, 2.9
21 mmol), benzylamine (310 mg, 2.9 mmol) and NaBH₄ (330 mg, 8.7 mmol) using the general procedure
22 for the synthesis of secondary amines (0.65 g, 85%). Rf = 0.3 (Hexane/AcOEt=9/1). ¹H NMR (300
23 MHz, CDCl₃): δ 7.63-7.60 (2H, d, J= 9 Hz, H arom.), 7.51-7.48 (2H, d, J= 8.3 Hz, H arom.), 7.36-7.30
24 (5H, m, H arom.), 3.89 (2H, s, -CH₂-trifluorobenz.), 3.84 (2H, s, -CH₂-benz.).

Mis en forme : Anglais (États-Unis)

1 **N-(4-Methoxybenzyl)-benzylamine (m).** Prepared from 4-methoxybenzaldehyde (500 mg, 3.7
2 mmol), benzylamine (393 mg, 3.7 mmol) and NaBH₄ (420 mg, 11.1 mmol) using the general procedure
3 for the synthesis of secondary amines (0.65 g, 77%). R_f = 0.3 (Hexane/AcOEt=9/1). ¹H NMR (300
4 MHz, CDCl₃): δ 7.39-7.31 (7H, m, H arom.), 6.92-6.89 (2H, m, H arom.), 3.82 (3H, s, -CH₃), 3.75 (2H,
5 s, -CH₂-methoxybenz.), 3.70 (2H, s, -CH₂-benz.).

6 **(2E)-N-Benzyl-3-phenylprop-2-en-amine (n).** Prepared from trans-cinnamaldehyde (660 mg, 5
7 mmol), benzylamine (535 mg, 5 mmol) and NaBH₄ (570 mg, 15 mmol) using the general procedure for
8 the synthesis of secondary amines (0.90 g, 81%). R_f = 0.35 (Hexane/AcOEt=8/2). ¹H NMR (300 MHz,
9 CDCl₃): δ 7.42-7.25 (10H, m, H arom.), 7.60-7.55 (1H, d, J= 15 Hz, H arom.), 6.40-6.30 (1H, m, -CH-),
10 3.88 (2H, s, -CH₂-benz.), 3.48-3.46 (2H, d, J= 6Hz, -CH₂-cinn.).

11 **N-(2-Naphthylmethyl)-3-aminopyridine (o).** To a solution of 2-naphtaldehyde (1eq, 500 mg,
12 3.2mmol) and 3-aminopyridine (1 eq, 300 mg, 3.2 mmol) in ethanol (0,3 M) heated at 95 °C during 48 h
13 then cooled to 0 °C, NaBH₄ (3 eq, 380 mg, 10 mmol) was added. The solution was stirred overnight at
14 RT and reduced. The resulting oil was dissolved in AcOEt; the organic layer was washed with a solution
15 of NaOH 1N (x2), water, saturated solution of NaCl, then dried and reduced. The resulting product was
16 purified by flash chromatography with AcOEt/Hexane (75/25) to yield an oil (300 mg, 40%). R_f = 0.3
17 (Hexane/AcOEt=1/9). ¹H NMR (300 MHz, CDCl₃): δ 8.15-8.11 (1H, d, H arom.), 7.97-7.94 (1H, m, H
18 arom.), 7.92-7.69 (4H, m, H arom.), 7.49-7.41 (3H, m, H arom.), 7.10-7.02 (1H, m, H arom.), 6.94-6.88
19 (1H, m, H arom.), 4.54-4.51 (2H, d, J= 8.9Hz, -CH₂-napht.).

20 **N-(2-Naphthylmethyl)-2-aminomethylpyridine (p).** Prepared from 2-naphtaldehyde (500 mg, 3.2
21 mmol), 2-aminomethylpyridine (350 mg, 3.2 mmol) and NaBH₄ (380 mg, 10 mmol) using the general
22 procedure for the synthesis of secondary amines (550 mg, 70%). The resulting oil was purified by flash
23 chromatography with AcOEt/Hexane/TEA (9/1/2%). R_f = 0,15 (Hexane/AcOEt/TEA=1/9/2%). ¹H
24 NMR (300 MHz, CDCl₃): δ 8.11-8.09 (1H, d, H arom.), 7.91-7.87 (4H, m, H arom.), 7.65-7.62 (1H, t, J
25 = 4.2 Hz, H arom.), 7.54-7.44 (3H, m, H arom.), 7.34-7.31 (1H, d, J = 7.8 Hz, H arom.), 7.19-7.15 (1H,
26 m, H arom.), 4.03 (2H, s, -CH₂-pyr.), 3.98 (2H, s, -CH₂-napht.).

1 **[N-Benzyl,N-(2-naphthylmethyl)-amino]-acetonitrile (1).** Prepared from the secondary amine **a**
2 (1.24 g, 5 mmol), chloroacetonitrile (415 mg, 5.5 mmol) and TEA (610 mg, 6 mmol) using the general
3 procedure for the synthesis of tertiary amines. The resulting product was purified in isopropyl alcohol to
4 yield a white solid (1.2 g, 84%). R_f = 0.3 (Hexane/AcOEt=9/1). ¹H NMR (300 MHz, CDCl₃): δ 7.90-
5 7.80 (4H, m, H arom.), 7.60-7.20 (8H, m, H arom.), 3.92 (2H, s, -CH₂-napht.), 3.81 (2H, s, -CH₂-benz.),
6 3.40 (2H, s, -CH₂-CN). ¹³C NMR (50 MHz, DMSO-d₆): δ 136.58, 134.80, 132.34, 132.20, 131.52,
7 130.87, 130.68, 113.81, 60.12, 59.20, 41.22. Mp = 92 °C; Anal. (C₁₈H₁₇N) C= 83.75%. H= 6.28%, N=
8 9.77%. HRMS m/z (M+H)⁺ 287.1538

9 **[4-Bromobenzyl-(2-naphthylmethyl)-amino]-acetonitrile (2).** Prepared from the secondary amine **b**
10 (3.42 g, 10.5 mmol), chloroacetonitrile (875 mg, 11.6 mmol) and TEA (1.27 g, 12.6 mmol) using the
11 general procedure for the synthesis of tertiary amines. The resulting product was purified by
12 crystallization (2.37 g, 65%). R_f = 0,15 (Hexane/AcOEt=9/1). ¹H NMR (200 MHz, CDCl₃): δ 7.90-7.85
13 (4H, m, H arom.), 7.55-7.51 (4H, m, H arom.), 7.36-7.30 (3H, m, H arom.), 3.92 (2H, s, -CH₂-napht.),
14 3.78 (2H, s, -CH₂-benz.), 3.42 (2H, s, -CH₂-CN). ¹³C NMR (50 MHz, DMSO-d₆): δ 136.57, 134.74,
15 133.70, 133.49, 132.26, 131.01, 129.01, 128.37, 128.22, 127.03, 126.77, 126.56, 122.15, 58.84, 58.08,
16 41.27. Mp = 110 °C. Anal. (C₁₈H₁₅BrN) C= 66.01%, H= 4.67%, N= 7.61%. HRMS m/z (M+H)⁺ 366.0648.

17 **[2-Bromobenzyl-(2-naphthylmethyl)-amino]-acetonitrile (3).** Prepared from the secondary amine **c**
18 (2.55 g, 7.8 mmol), chloroacetonitrile (648 mg, 8.6 mmol) and TEA (945 mg, 9.36 mmol) using the
19 general procedure for the synthesis of tertiary amines. The resulting product was purified by flash
20 chromatography with Hexane/AcOEt=9/1 to yield a white solid (1,7 g, 60%). R_f = 0.9
21 (Hexane/AcOEt=2/1). ¹H NMR (200 MHz, CDCl₃): δ 7.88-7.84 (3H, m, H arom.), 7.68-7.50 (4H, m, H
22 arom.), 7.41-7.18 (4H, m, H arom.), 3.99 (2H, s, -CH₂-napht.), 3.97 (2H, s, -CH₂-bromobenz.), 3.41
23 (2H, s, -CH₂-CN). ¹³C NMR (75 MHz, DMSO-d₆): δ 132.91, 131.80, 129.5, 127.72, 127.0, 126.49,
24 126.32, 58.76, 57.97, 41.45. Mp = 71 °C. Anal. (C₁₈H₁₅BrN) C= 63.0%, H= 4.72%, N= 7.22%, 1 H₂O.
25 HRMS m/z (M+H)⁺ 366.0648.

1 **[4-Iodobenzyl-(2-naphthylmethyl)-amino]-acetonitrile (4).** Prepared from the secondary amine **d**
2 (4.83 g, 12.8 mmol), chloroacetonitrile (982 mg, 13 mmol) and TEA (1.31 g, 13 mmol) using the
3 general procedure for tertiary amines. The resulting product was purified by flash chromatography with
4 Hexane/AcOEt=95/5 to yield a white solid (3.65 g, 69%). Rf = 0.25 (Hexane/AcOEt=95/5). ¹H NMR
5 (200 MHz, CDCl₃): δ 7.90-7.85 (4H, m, H arom.), 7.55-7.51 (4H, m, H arom.), 7.36-7.30 (3H, m, H
6 arom.), 3.92 (2H, s, -CH₂-napht.), 3.78 (2H, s, -CH₂-iodobenz.), 3.42 (2H, s, -CH₂-CN). ¹³C NMR (75
7 MHz, DMSO-d₆): δ 137.66, 135.27, 133.23, 132.88, 131.33, 128.57, 127.90, 127.63, 127.02, 126.59,
8 126.32, 115.78, 93.85, 57.75, 57.09, 41.50. Mp = 116 °C. HRMS m/z (M+H)⁺ 413.0509.

9 **[4-Phenethylbenzyl-(2-naphthylmethyl)-amino]-acetonitrile (5).** Prepared from the secondary
10 amine **e** (200 mg, 0.57 mmol), chloroacetonitrile (43 mg, 0.57 mmol) and TEA (57 mg, 0.57 mmol)
11 using the general procedure for the tertiary amine. The resulting product was purified by flash
12 chromatography with Hexane/AcOEt=95/5 to yield a white solid (195 mg, 87%). Rf = 0.15
13 (Heptane/AcOEt=95/5). ¹H NMR (300 MHz, CDCl₃): δ 7.91-7.87 (4H, m, H arom.), 7.60-7.51 (3H, m,
14 H arom.), 7.40-7.20 (9H, m, H arom.), 3.94 (2H, s, -CH₂-napht.), 3.81 (2H, s, -CH₂-benz.), 3.43 (2H, s, -
15 CH₂-CN), 2.96 (4H, s, -CH₂-CH₂-). ¹³C NMR (75 MHz, CDCl₃): δ 141.94, 140.90, 136.57, 133.02,
16 131.47, 130.97, 128.54, 125.01, 119.78, 113.82, 63.31, 45.83, 37.44. HRMS m/z (M+H)⁺ 391.47

17 **[4-Methoxybenzyl-(2-naphthylmethyl)-amino]-acetonitrile (6).** Prepared from the secondary amine
18 **f** (720 mg, 2.6 mmol), chloroacetonitrile (212 mg, 2.8 mmol) and TEA (323 mg, 3.2 mmol) using the
19 general procedure for tertiary amines. The resulting product was purified by flash chromatography with
20 Hexane/AcOEt=95/5 to yield a white solid (595 mg, 60%). Rf = 0.45 (Hexane/AcOEt=8/2). ¹H NMR
21 (200 MHz, CDCl₃): δ 7.86-7.82 (4H, m, H arom.), 7.54-7.46 (3H, m, H arom.), 7.36-7.32 (2H, m, H
22 arom.), 3.89 (2H, s, -CH₂-napht.), 3.82 (3H, s, -CH₃), 3.74 (2H, s, -CH₂-benz.), 3.38 (2H, s, -CH₂-CN).
23 ¹³C NMR (50 MHz, DMSO-d₆): δ 154.44, 129.86, 128.47, 128.20, 125.40, 124.22, 123.65, 123.03,
24 122.97, 122.86, 121.97, 121.43, 121.20, 109.88, 109.21, 53.50, 52.95, 50.46, 35.77. Mp= 86 °C. Anal.
25 (C,H,N) C= 79.45%, H= 6.54%, N= 8.74%. HRMS m/z (M+H)⁺ 317.1648.

1 **[Benzyl-(3-chlorobenzyl)-amino]-acetonitrile hydrochloride (7).** Prepared from the secondary
2 amine **g** (1.50 g, 6.40 mmol), chloroacetonitrile (530 mg, 7.0 mmol) and TEA (775 mg, 7.7 mmol) using
3 the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash
4 chromatography with Hexane/AcOEt=95/5 to yield a white solid (805 mg, 41%). R_f = 0.3
5 (Hexane/AcOEt=95/5). ^1H NMR (300 MHz, CDCl_3): δ 7.81-7.78 (4H, m, H arom.), 7.51-7.46 (5H, m,
6 H arom.), 4.30 (2H, s, $-\text{CH}_2\text{-chlorobenz.}$), 4.27 (2H, s, $-\text{CH}_2\text{-benz.}$), 3.75 (2H, s, $-\text{CH}_2\text{-CN}$). ^{13}C NMR
7 (50 MHz, DMSO-d_6): δ 137.54, 135.03, 133.71, 133.46, 129.41, 129.10, 128.92, 128.25, 128.10,
8 127.14, 126.70, 126.46, 115.08, 58.87, 58.82, 41.22. Synthesis of the salt. Mp = 120 °C. Anal. (C,H,N)
9 C= 62.40%, H= 5.47%, N= 8.14% 1 HCl. HRMS m/z ($\text{M}+\text{H}$) $^+$ 271.1002.

10 **[Benzyl-(4-chlorobenzyl)-amino]-acetonitrile hydrochloride (8).** Prepared from the secondary
11 amine **h** (1.05 g, 4.5 mmol), chloroacetonitrile (375 mg, 4.95 mmol) and TEA (545 mg, 5.4 mmol) using
12 the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash
13 chromatography with Hexane/AcOEt=95/5 to yield a white solid (580 mg, 42%). R_f = 0.3
14 (Hexane/AcOEt=95/5). ^1H NMR (300 MHz, CDCl_3): δ 7.79-7.76 (4H, m, H arom.), 7.50-7.45 (5H, m,
15 H arom.), 4.32 (2H, s, $-\text{CH}_2\text{-chlorobenz.}$), 4.28 (2H, s, $-\text{CH}_2\text{-benz.}$), 3.73 (2H, s, $-\text{CH}_2\text{-CN}$). ^{13}C NMR
16 (75 MHz, DMSO-d_6): δ 136.95, 136.17, 132.58, 131.00, 129.20, 128.92, 128.89, 128.09, 115.51, 57.52,
17 56.76, 41.25. Mp = 124 °C. Anal. (C,H,N) C= 62.83%, H= 5.24%, N= 9.12%, 1 HCl. HRMS m/z
18 ($\text{M}+\text{H}$) $^+$ 271.0984.

19 **[Benzyl-(1H-indol-3-ylmethyl)-amino]-actonitrile hydrochloride (9).** Prepared from the secondary
20 amine **i** (613 mg, 2.6 mmol), chloroacetonitrile (216 mg, 2.85 mmol) and TEA (315 g, 3.12 mmol) using
21 the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash
22 chromatography with Hexane/AcOEt=2/1 to yield a white solid (640 mg, 80%). R_f = 0.75
23 (Hexane/AcOEt=2/1). ^1H NMR (300 MHz, CDCl_3): δ 7.81-7.76 (1H, d, J = 5 Hz, H arom.), 7.45-7.30
24 (7H, m, H arom.), 7.25-7.16 (2H, m, H arom.), 3.97 (2H, s, $-\text{CH}_2\text{-indol.}$), 3.82 (2H, s, $-\text{CH}_2\text{-benz.}$), 3.39
25 (2H, s, $-\text{CH}_2\text{-CN}$). ^{13}C NMR (75 MHz, DMSO-d_6): δ 139.41, 137.12, 131.94, 131.13, 129.54, 128.07,

123.60, 122.47, 120.23, 119.48, 114.75, 112.18, 102.21, 50.74, 50.06, 34.06. Synthesis of the salt. Mp = 150 °C. Anal. (C,H,N) C= 68.71%, H= 5.81%, N= 13.49%, 1HCl. HRMS m/z (M+H)⁺ 276.5005.

[Benzyl-(quinolin-2-ylmethyl)-amino]-acetonitrile dihydrochloride (10). Prepared from the secondary amine **j** (635 mg, 2.56 mmol), chloroacetonitrile (212 mg, 2.81 mmol) and TEA (310 g, 3.07 mmol) using the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash chromatography with Hexane/AcOEt=8/2 to afford a yellow solid (690 mg, 75%). Rf = 0.2 (Hexane/AcOEt=8/2). ¹H NMR (300 MHz, CDCl₃): δ 7.85-7.83 (1H, d, H arom.), 7.82-7.80 (1H, d, H arom.), 7.74-7.72 (1H, d, H arom.), 7.63-7.56 (1H, d, H arom.), 7.44-7.37 (2H, m, H arom.), 7.35-7.31 (5H, m, H arom.), 4.11 (2H, s, -CH₂-quinol.), 3.83 (2H, s, -CH₂-benz.), 3.55 (2H, s, -CH₂-CN). ¹³C NMR (75 MHz, DMSO-d₆): δ 157.79, 144.34, 140.06, 136.94, 133.69, 129.35, 128.82, 128.06, 127.93, 122.65, 122.13, 116.13, 58.35, 56.28, 42.72. Synthesis of the salt. Mp = 160 °C. Anal. (C,H,N) C= 63.53%, H= 5.35%, N= 11.59%, 2HCl. HRMS m/z (M+H)⁺ 288.1488.

[Benzyl-(quinolin-3-ylmethyl)-amino]-acetonitrile dihydrochloride (11). Prepared from the secondary amine **k** (635 mg, 2.56 mmol), chloroacetonitrile (210 mg, 2.80 mmol) and TEA (310 g, 3.07 mmol) using the general procedure for the synthesis of tertiary amine. The resulting product was purified by flash chromatography with Hexane/AcOEt=7/3 to yield a yellow solid (505 mg, 55%). Rf = 0.3 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 7.83-7.81 (1H, d, H arom.), 7.84-7.81 (1H, d, H arom.), 7.75-7.73 (1H, d, H arom.), 7.63-7.55 (1H, d, H arom.), 7.44-7.37 (2H, m, H arom.), 7.35-7.29 (5H, m, H arom.), 4.10 (2H, s, -CH₂-quinol.), 3.85 (2H, s, -CH₂-benz.), 3.57 (2H, s, -CH₂-CN). ¹³C NMR (75 MHz, DMSO-d₆): δ 148.51, 140.33, 138.15, 133.92, 131.45, 130.97, 126.71, 124.61, 123.64, 123.19, 117.92, 61.54, 57.96, 44.64. Synthesis of the salt. Mp = 190 °C. Anal. (C,H,N) C= 63.54%, H= 5.36%, N= 11.79%, 2HCl. HRMS m/z (M+H)⁺ 288.1509.

{Benzyl-[(2E)-3-phenylprop-2-en-1-yl]-amino}-acetonitrile hydrochloride (12). Prepared from the secondary amine **n** (903 mg, 4.05 mmol), chloroacetonitrile (336 mg, 4.45 mmol) and TEA (490 g, 4.85 mmol) using the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash chromatography with Hexane/AcOEt=9/1 to yield a yellow solid (556 mg, 46%). Rf =

0,3 (Hexane/AcOEt=9/1). ¹H NMR (200 MHz, CDCl₃): δ = 7.49-7.28 (10H, m, H arom.), 6.73-6.65 (1H, d, J = 16 Hz, H arom.), 6.29-6.14 (1H, m, -CH-), 3.75 (2H, s, -CH₂-benz.), 3.51 (2H, s, -CH₂-cinn.), 3.43-3.39 (2H, d, J = 6.8 Hz, -CH₂-CN). ¹³C NMR (75 MHz, DMSO-d₆): δ = 136.28, 136.16, 134.23, 130.26, 129.03, 128.98, 128.48, 127.00, 122.40, 114.79, 57.31, 55.68, 41.58. Synthesis of the salt. Mp = 170 °C. Anal. (C₁₈H₁₇N) C= 71.96%, H= 6.49%, N= 9.18%, 1HCl. HRMS m/z (M+H)⁺ 263.1544.

[Benzyl-(4-methoxybenzyl)-amino]-acetonitrile hydrochloride (13). Prepared from the secondary amine **m** (635 mg, 2.85 mmol), chloroacetonitrile (237 mg, 3.14 mmol) and TEA (345 mg, 3.42 mmol) using the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash chromatography with Hexane/AcOEt=97/3 to yield a white solid (485 mg, 56%). R_f = 0.2 (Hexane/AcOEt=97/3). ¹H NMR (300 MHz, CDCl₃): δ 7.39-7.31 (7H, m, H arom.), 6.92-6.89 (2H, m, H arom.), 3.82 (3H, s, -CH₃), 3.74 (2H, s, -CH₂-methoxybenz.), 3.70 (2H, s, -CH₂-benz.), 3.37 (2H, s, -CH₂-CN). ¹³C NMR (50 MHz, DMSO-d₆): δ 161.54, 133.27, 131.68, 130.86, 130.01, 128.85, 120.43, 115.30, 111.65, 58.54, 58.45, 55.81, 38.75. Synthesis of the salt. Mp = 114 °C. HRMS m/z (M+H)⁺ 267.1485.

{Benzyl-[4-(trifluoromethyl)-benzyl]-amino}-acetonitrile hydrochloride (14). Prepared from the secondary amine **l** (660 mg, 2.5 mmol), chloroacetonitrile (210 mg, 2.75 mmol) and TEA (300 mg, 3 mmol) using the general procedure for the synthesis of tertiary amines. The resulting product was purified by flash chromatography with Hexane/AcOEt=97/3 to yield a white solid (580 mg, 68%). R_f = 0.3 (Hexane/AcOEt=97/3). ¹H NMR (300 MHz, CDCl₃): δ 7.72-7.68 (2H, d, H arom.), 7.60-7.55 (2H, m, H arom.), 7.41-7.35 (5H, m, H arom.), 3.83 (2H, s, -CH₂-trifluorobenz.), 3.78 (2H, s, -CH₂-benz.), 3.41 (2H, s, -CH₂-CN). ¹³C NMR (75 MHz, DMSO-d₆): δ 151.36, 147.16, 140.13, 133.21, 132.82, 131.76, 131.12, 129.33, 128.95, 128.52, 127.94, 127.21, 126.92, 124.76, 57.85, 54.77. Synthesis of the salt. HRMS m/z (M+H)⁺ 305.1261.

1 **[Benzyl-(2-naphthylmethyl)-propargyl]-amine (15).** Prepared from the secondary amine **a** (1.24 g,
2 5 mmol), propargyl bromide (655 mg, 5.5 mmol) and TEA (610 mg, 6 mmol) using the general
3 procedure for the synthesis of tertiary amines. The resulting product was purified in isopropyl alcohol to
4 yield a white solid (1.29 g, 82%). R_f = 0.3 (Hexane/AcOEt=9/1). ¹H NMR (300 MHz, CDCl₃): δ 7.90-
5 7.80 (4H, m, H arom.), 7.60-7.20 (8H, m, H arom.), 3.93 (2H, s, -CH₂-napht.), 3.82 (2H, s, -CH₂-benz.),
6 3.34 (2H, s, -CH₂-CN). ¹³C NMR (50 MHz, DMSO-d₆): δ 136.58, 134.80, 132.34, 132.20, 131.52,
7 130.87, 130.68, 113.81, 60.12, 59.20, 41.22. Mp = 92 °C. Anal. (C₂₁H₁₉N) C= 88.40%, H= 6.68%, N=
8 4.98%. HRMS m/z (M+H)⁺ 286.1538.

9 **4-[Benzyl-(2-naphthylmethyl)-amino]-butyronitrile hydrochloride (16).** Prepared from the
10 secondary amine **a** (500 mg, 2.5 mmol), 4-bromobutyronitrile (410 mg, 2.75 mmol) and TEA (303 mg, 3
11 mmol) using the general procedure for tertiary amines. The resulting product was purified by flash
12 chromatography with Hexane/AcOEt=95/5 to yield a yellow solid (550 mg, 63%). R_f= 0.2
13 (Hexane/AcOEt=9/1). ¹H NMR (300 MHz, CDCl₃): δ 7.90-7.81 (3H, m, H arom.), 7.75 (1H, s, H
14 arom.), 7.49-7.46 (3H, m, H arom.), 7.37-7.35 (5H, m, H arom.), 3.73 (2H, s, -CH₂-napht.), 3.63 (2H, s,
15 -CH₂-benz.), 2.60 (2H, t, J = 6.4 Hz, -CH₂-N), 2.32 (2H, t, J = 7.5 Hz, -CH₂-CN), 1.85-1.81 (2H, m, -
16 CH₂-CH₂-CH₂-CN). ¹³C NMR (50 MHz, DMSO-d₆): δ 133.38, 132.88, 131.88, 131.69, 130.16, 129.83,
17 129.83, 129.16, 128.75, 128.57, 128.44, 128.00, 127.67, 127.44, 127.02, 119.91, 56.55, 50.13, 19.62,
18 14.25. Synthesis of the salt. Anal. (C₂₁H₁₉N) C= 71.46%, H= 6.70%, N= 7.47%, 1HCl, 1H₂O. HRMS m/z
19 (M+H)⁺ 315.1850.

20 **N-Ethyl-[benzyl-(2-naphthylmethyl)-amine] acetate (17).** Prepared from the secondary amine **a**
21 (800 mg, 4 mmol), 2-bromoethylacetate (740 mg, 4.5 mmol) and TEA (505 mg, 5 mmol) using the
22 general procedure for tertiary amines. The resulting product was purified by flash chromatography with
23 Hexane/AcOEt=95/5 to yield a white solid (1.12 g, 84%). R_f = 0.25 (Hexane/AcOEt=95/5). ¹H NMR
24 (200 MHz, CDCl₃): δ 7.90-7.78 (4H, m, H arom.), 7.60-7.55 (1H, d, H arom.), 7.48-7.66 (7H, m, H
25 arom.), 4.18-4.14 (2H, q, -CH₂-CH₃), 3.98 (2H, s, -CH₂-napht.), 3.87 (2H, s, -CH₂-benz.), 3.32 (2H, s, -

1 CH₂-CN), 1.27 (3H, t, J= 4.5Hz, -CH₃). ¹³C NMR (50 MHz, DMSO-d₆): δ 170.89, 139.13, 136.92,
2 133.26, 132.76, 128.98, 127.89, 127.87, 127.38, 126.39, 126.01, 60.10, 57.58, 57.36, 53.54, 14.44. Mp
3 = 69 °C. Anal. (CHN) C= 71.46%, H= 6.70%, N= 3.47%, HCl. HRMS m/z (M+H)⁺ 334.1802.

4 **[Benzyl-(2-naphthylmethyl)-amino]-acetic acid (18)**. Prepared from the tertiary amine **17** (400 mg,
5 1.7 mmol) and HCl 6N (12 mL) heated at 100°C over night. The solution was cooled and the precipitate
6 was filtered then washed with few mL of diethylether to yield a white solid (510 mg, 98%). ¹H NMR
7 (300 MHz, DMSO-d₆): δ 8.05 (1H, s, H arom.), 7.98-7.92 (3H, m, H arom.), 7.72-7.68 (1H, d, H
8 arom.), 7.58-7.54 (4H, m, H arom.), 7.43-7.40 (3H, m, H arom.), 4.49 (2H, s, -CH₂-COOH), 4.38 (2H, s,
9 -CH₂-napht.), 3.76 (2H, s, -CH₂-benz.). ¹³C NMR (50 MHz, DMSO-d₆): δ 170.21, 134.97, 133.34,
10 132.98, 131.91, 130.91, 129.74, 129.27, 128.55, 121.13, 60.37, 60.09, 52.91. Mp = 154 °C. Anal.
11 (C,H,N) C= 79.32%, H= 6.99%, N= 4.19%. HRMS m/z (M+H)⁺ 306.1489.

12 **Ethyl 5-[Benzyl-(2-naphthylmethyl)-amino]-pentanoate (19)**. Prepared from the secondary amine **a**
13 (1 g, 4 mmol), 5-bromoethylvalerate (836 mg, 4 mmol) and TEA (808 mg, 8 mmol). The resulting
14 product was purified by flash chromatography with Hexane/AcOEt=95/5 to yield a white solid (1.07 g,
15 70%). R_f = 0.15 (Heptane/AcOEt=95/5). ¹H NMR (200 MHz, CDCl₃): δ 7.89-7.77 (4H, m, H arom.),
16 7.48-7.32 (8H, m, H arom.), 4.19-4.05 (2H, m, -CH₂-napht.), 3.71 (2H, s, -CH₂-benz.), 3.60 (2H, s, -
17 CH₂-CH₂-CH₂-CH₂-COOEt), 2.51-2.45 (2H, m, -CH₂-COOEt), 2.37-2.30 (2H, m, -CH₂-CH₃), 1.62-
18 1.58 (4H, m, -CH₂-CH₂-CH₂-CH₂-COOEt), 1.30-1.24 (3H, m, -CH₃). ¹³C NMR (50 MHz, DMSO-d₆): δ
19 174.32, 133.28, 132.92, 131.78, 130.32, 129.79, 128.54, 127.97, 127.42, 127.00, 56.31, 50.98, 45.63,
20 31.04, 22.35, 8.74. Mp = 126 °C. MS m/z (M+H)⁺ 376.16.

21 **5-[benzyl2-naphthylmethyl)-amino]-pentanoic acid hydrochloride (20)**. A solution of tertiary
22 amine **18** (100 mg, 0.27 mmol) in HCl 6N (12 mL) was heated overnight at 100 °C and then cooled at 0
23 °C. The precipitate was filtered and suspended in diethylether to yield a white solid (105 mg,100%). ¹H
24 NMR (300 MHz, DMSO-d₆): δ 8.16-7.84 (4H, m, H arom.), 7.67-7.58 (4H, m, H arom.), 7.45-7.39 (4H,
25 m, H arom.), 4.20-4.15 (2H, m, -CH₂-napht.), 3.81-3.79 (2H, m, -CH₂-benz.), 3.70-3.68 (2H, m, -CH₂-

1 CH₂-CH₂-CH₂-COOH), 2.51-2.45 (2H, m, -CH₂-COOH), 1.62-1.58 (4H, m, -CH₂-CH₂-CH₂-CH₂-
2 COOH). ¹³C NMR (50 MHz, DMSO-d₆): δ 174.30, 133.34, 132.87, 131.78, 131.59, 130.33, 129.79,
3 129.13, 128.69, 128.53, 128.41, 128.00, 127.80, 127.43, 127.02, 56.28, 50.98, 33.19, 31.04, 22.36,
4 21.97. Mp = 84 °C. MS m/z (M+H)⁺ 348.06.

5 **[Benzyl-(2-naphthylmethyl)-amino]-hexadecanoyl acetate hydrochloride (21)**. Prepared from 1-
6 hexadecanol (187 mg, 0.77 mmol), acid **18** (200 mg, 0.65 mmol), BOP (345 mg, 0.78 mmol) and NMM
7 (303 mg, 3 mmol) in a solution of acetonitrile 5 mL and DMF 500 μL using the general procedure for
8 the synthesis of esters. The resulting product was purified by flash chromatography with
9 Hexane/AcOEt=98/2 to yield an oil (221 mg, 60%). R_f = 0.5 (Heptane/AcOEt: 95/5). ¹H NMR (300
10 MHz, CDCl₃): δ 7.89-7.75 (4H, m, H arom.), 7.51-7.32 (8H, m, H arom.), 4.17-4.11 (2H, t, J = 6 Hz, -
11 CH₂-O-), 4.02 (2H, s, -CH₂-napht.), 3.91 (2H, s, -CH₂-benz.), 3.37 (2H, s, -CH₂-CO), 1.30 (31H, m, -
12 (CH₂)₁₄-CH₃). ¹³C NMR (50 MHz, CDCl₃): δ 166.39, 134.18, 133.42, 132.12, 130.65, 129.95, 129.41,
13 128.75, 128.25, 127.82, 127.25, 126.86, 66.89, 58.07, 48.05, 32.32, 30.09, 29.84, 29.77, 29.49, 28.65,
14 26.06, 23.03, 14.53. Synthesis of the salt. Mp = 140 °C. HRMS m/z (M+H)⁺ 530.3993.

15 **[Benzyl-(2-naphthylmethyl)-amino]-phenethyle acetate hydrochloride (22)**. Prepared from
16 phenethyl alcohol (81 mg, 0.77 mmol), acid **18** (200 mg, 0.65 mmol), BOP (345 mg, 0.78 mmol) and
17 NMM (303 mg, 3 mmol) in a solution of acetonitrile 5 mL and DMF 500 μL using the general
18 procedure for the synthesis of esters. The resulting product was purified by flash chromatography with
19 Hexane/AcOEt=95/5 to yield a yellow hygroscopic powder (198 mg, 57%). R_f = 0.25
20 (Heptane/AcOEt=95/5). ¹H NMR (300 MHz, CDCl₃): δ 7.79-7.74 (3H, m, H arom.), 7.62 (1H, s, H
21 arom.), 7.51-7.47 (2H, m, H arom.), 7.36-7.14 (11H, m, H arom.), 4.86 (2H, t, -CH₂-O-), 3.68 (2H, s, -
22 CH₂-napht.), 3.57 (2H, s, -CH₂-benz.), 3.21 (2H, s, -CH₂-CO-), 2.97 (2H, t, -CH₂-Phen.). ¹³C NMR (50
23 MHz, CDCl₃): δ 166.17, 136.96, 134.20, 133.43, 132.32, 132.16, 130.69, 129.86, 129.24, 129.13,
24 128.85, 128.25, 127.85, 127.35, 126.65, 67.06, 58.21, 48.77, 35.20. HRMS m/z (M+H)⁺ 410.2115.

1 **Methylsulfonyl 2-hydroxyethyl (q).** In a solution of DCM (50 mL) with TEA (4,9 g, 48.3 mmol)
2 and ethylene glycol (2 g, 32.3 mmol) was added mesyl chloride (5.53 mg, 48.03 mmol) at 0 °C during 3
3 h, then stirred 2 h at RT. The resulting product was purified by flash chromatography with
4 Hexane/AcOEt=25/75 to yield a colorless oil (905 mg, 20%). Rf = 0.2 (Heptane/AcOEt=25/75). ¹H
5 NMR (300 MHz, CDCl₃): δ 4.37-4.34 (2H, m, -CH₂-O-SO₂), 3.92-3.89 (2H, m, -CH₂-OH), 3.09 (3H, s,
6 -CH₃), 2.20 (1H, s, -OH).

7 **Methylsulfonyl ethyl-[benzyl-(2-naphthylmethyl)-amino]-2-acetate (r).** To a solution of acid **18**
8 (200 mg, 0.65 mmol) in acetonitrile 5 mL and DMF 500 μL was added NMM (303 mg, 3 mmol), BOP
9 (292 mg, 0.66 mmol) and ethylene glycol monomesyle (100 mg, 0.7 mmol). The mixture was stirred at
10 RT overnight. The resulting product was purified by flash chromatography with Hexane/AcOEt (from
11 9/1 to 7/3) to yield a white oil (98 mg, 35%). Rf = 0.4 (Heptane/AcOEt=25/75). ¹H NMR (300 MHz,
12 CDCl₃): δ 7.92-7.80 (4H, m, H arom.), 7.63-7.55 (1H, m, H arom.), 7.44-7.28 (7H, m, H arom.), 4.38-
13 4.33 (4H, m, -CH₂-CH₂-), 3.98 (2H, s, -CH₂-napht.), 3.87 (2H, s, -CH₂-benz.), 3.39 (2H, s, -CH₂-CO),
14 2.93 (3H, s, -CH₃).

15 **[Benzyl-(2-naphthylmethyl)-amino]-2-hydroxyethyl acetate (23).** The mesyl **r** (100 mg, 0.23
16 mmol) was dissolved in toluene 10 mL, then were added few mg of para-toluene sulfonic acid and the
17 mixture was stirred at reflux during 16 h. The resulting product was purified by flash chromatography
18 with Hexane/AcOEt=8/2 to yield a yellow powder (30 mg, 35%). ¹H NMR (300 MHz, CDCl₃): δ 7.92-
19 7.79 (4H, m, H arom.), 7.63-7.57 (3H, m, H arom.), 7.45-7.28 (5H, m, H arom.), 4.38-4.33 (2H, m, -
20 CH₂-OCO-), 4.15-4.10 (2H, m, -CH₂-OH), 3.98 (2H, s, -CH₂-napht.), 3.93 (2H, s, -CH₂-benz.), 3.19
21 (2H, s, -CH₂-CO). ¹³C NMR (50 MHz, DMSO-d₆): δ 170.72, 140.39, 132.94, 129.04, 128.62, 128.35,
22 127.96, 126.89, 124.89, 123.78, 119.78, 59.98, 58.91, 57.94, 56.87. MS m/z (M+H)⁺ 350.3.

23 **[Benzyl-(2-naphthylmethyl)-amino]-ethylmorpholine acetate hydrochloride (24).** Prepared from
24 4-(2-hydroxyethyl)morpholine (101 mg, 0.77 mmol), acid **18** (200 mg, 0.65 mmol), BOP (345 mg, 0.78
25 mmol) and NMM (303 mg, 3 mmol) in a solution of acetonitrile 5 mL and THF 500 μL using the
26 general procedure for the synthesis of esters. The resulting product was purified by flash

1 chromatography with Hexane/AcOEt (from 7/3 to 5/5) to yield an oil (118 mg, 40%). Rf = 0.3
2 (Heptane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 7.89-7.79 (4H, m, H arom.), 7.65-7.60 (1H, m, H
3 arom.), 7.47-7.30 (7H, m, H arom.), 4.30-4.26 (2H, t, J= 4Hz, -O-CH₂-CH₂-), 4.01 (2H, s, -CH₂-napht.),
4 3.90 (2H, s, -CH₂-benz.), 3.72-3.68 (4H, t, J = 4 Hz, -CH₂-O-CH₂-), 3.38 (2H, s, -CH₂-CO), 2.68-2.62
5 (2H, t, J = 6 Hz, -CH₂-N), 2.54-2.49 (4H, m, -CH₂-N-CH₂-). ¹³C NMR (50 MHz, DMSO-d₆): δ 167.72,
6 133.43, 132.85, 131.84, 131.67, 130.41, 129.92, 129.15, 128.73, 128.47, 128.01, 127.47, 127.03, 63.40,
7 58.52, 57.73, 55.27, 51.78, 50.92, 31.06. Synthesis of the salt. HRMS m/z (M+H)⁺ 419.2329.

8 **[Benzyl-(2-naphthylmethyl)-amino]-N-butyl-acetamide hydrochloride (25).** Prepared from n-
9 butylamine (45 mg, 0.62 mmol), acid **18** (200 mg, 0.59 mmol), BOP (273 mg, 0.62 mmol) and NMM
10 (130 mg, 1.29 mmol) using the general procedure for the synthesis of amides. The resulting product was
11 purified by flash chromatography with Hexane/AcOEt=7/3 to yield a white powder (200 mg, 85%). Rf=
12 0.2 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 7.91-7.88 (3H, m, H arom.), 7.75 (1H, s, H
13 arom.), 7.38-7.36 (3H, m, H arom.), 7.35-7.30 (5H, m, H arom.), 3.79 (2H, s, -CH₂-napht.), 3.69 (2H, s,
14 -CH₂-benz.), 3.18 (2H, s, -CH₂-CO), 1.48-1.40 (2H, m, -NH-CH₂-), 1.35-1.25 (2H, m, -CH₂-CH₂-CH₂-
15 CH₃), 0.97-0.88 (5H, m, -CH₂-CH₃). ¹³C NMR (50 MHz, DMSO-d₆): δ 165.76, 135.72, 134.91, 133.37,
16 132.95, 131.85, 130.85, 130.63, 129.77, 129.27, 129.08, 128.48, 128.04, 60.91, 60.75, 53.29, 40.59,
17 32.45, 21.27, 14.31. Synthesis of the salt. Mp = 160 °C. HRMS m/z (M+H)⁺ 361.2274.

18 **[Benzyl-(2-naphthylmethyl)-amino]-piperidino-acetamide hydrochloride (26).** Prepared from
19 piperidine (52 mg, 0.62 mmol), acid **18** (200 mg, 0.59 mmol), BOP (273 mg, 0.62 mmol) and NMM
20 (130 mg, 1.29 mmol) using the general procedure for the synthesis of amides. The resulting product was
21 purified by flash chromatography with Hexane/AcOEt=8/2 to yield a white powder (220 mg, 91%). ¹H
22 NMR (300 MHz, CDCl₃): δ 7.85-7.75 (4H, m, H arom.), 7.57-7.48 (3H, m, H arom.), 7.41-7.29 (5H, m,
23 H arom.), 3.85 (2H, s, -CH₂-napht.), 3.74 (2H, s, -CH₂-benz.), 3.51-3.47 (2H, t, J = 5.28 Hz), 3.33 (2H,
24 s, -CH₂-CO), 3.24-3.21 (2H, t, J = 5.28 Hz, -N-CH₂), 1.61-1.51 (4H, m, -N-CH₂-CH₂-), 1.46-1.42 (2H,
25 m, -CH₂-piper.). ¹³C NMR(50 MHz, DMSO-d₆): δ 180.02, 145.90, 132.79, 131.97, 129.10, 128.63,

128.43, 127.97, 127.00, 58.93, 45.51, 42.66, 25.43, 25.00, 23.77. Synthesis of the salt. HRMS m/z (M+H)⁺ 373.2274.

[Benzyl-(2-naphthylmethyl)-amino]-(N'-methyl)-piperazido-acetamide hydrochloride (27).

Prepared from N-methylpiperazine (62 mg, 0.62 mmol), acid **18** (200 mg, 0.59 mmol), BOP (273 mg, 0.62 mmol) and NMM (130 mg, 1.29 mmol) using the general procedure for the synthesis of amides. The resulting product was purified by flash chromatography with AcOEt/MeOH/TEA (9/1/2%) to yield a white powder (168 mg, 67%). R_f = 0.3 (AcOEt/MeOH/TEA=9/1/2%). ¹H NMR (300 MHz, CDCl₃): δ 7.91-7.88 (3H, m, H arom.), 7.77 (1H, s, H arom.), 7.54-7.48 (3H, m, H arom.), 7.37-7.32 (5H, m, H arom.), 3.87 (2H, s, -CH₂-napht.), 3.76 (2H, s, -CH₂-benz.), 3.61-3.55 (2H, t, J= 4.9Hz, -CH₂-NCO), 3.36-3.32 (4H, m, -CH₂-CO-N-CH₂-), 2.38-2.29 (7H, m, -CH₂-NCH₃-CH₂-). ¹³C NMR (50 MHz, DMSO-d₆): δ 164.99, 135.64, 134.82, 133.52, 133.05, 131.88, 130.53, 129.87, 129.36, 129.21, 128.64, 128.29, 61.11, 54.10, 52.98, 44.03, 43.21, 40.40. Synthesis of the salt. HRMS m/z (M+H)⁺ 388.2383.

[Benzyl-(2-naphthylmethyl)-amino]-(N'-phenyl)-piperazido-acetamide hydrochloride (28).

Prepared from N-phenylpiperazine (100 mg, 0.65 mmol), acid **18** (200 mg, 0.59 mmol), BOP (273 mg, 0.62 mmol) and NMM (130 mg, 1.29 mmol) using the general procedure for the synthesis of amides. The resulting product was purified by flash chromatography with Hexane/AcOEt=7/3 to yield a white powder (240 mg, 84%). R_f = 0.25 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 7.92-7.87 (3H, m, H arom.), 7.77 (1H, s, H arom.), 7.54-7.47 (3H, m, H arom.), 7.37-7.32 (7H, m, H arom.), 6.95-6.89 (3H, m, H arom.), 3.87 (2H, s, -CH₂-napht.), 3.76 (2H, s, -CH₂-benz.), 3.74-3.72 (2H, m, -CH₂-N-phenyl), 3.47-3.45 (2H, m, -CH₂-N-phenyl), 3.35 (2H, s, -CH₂-CO), 3.13-3.10 (2H, t, J= 5.3Hz, -CO-NH-CH₂-), 3.03-3.00 (2H, t, J = 5.0 Hz, -CO-NH-CH₂-). ¹³C NMR (50 MHz, DMSO-d₆): δ 163.27, 149.35, 133.48, 132.78, 132.09, 131.96, 130.04, 129.48, 129.14, 128.73, 128.66, 128.48, 127.97, 127.55, 127.03, 121.55, 117.20, 58.82, 50.47, 49.06, 43.99, 43.99, 41.07. Synthesis of the salt. Mp = 100 °C. HRMS m/z (M+H)⁺ 450.2540.

[Benzyl-(2-naphthylmethyl)-amino]-3-pyridine hydrochloride (29). A solution of secondary amine

p (234 mg, 1 mmol), and NaH (48 mg, 2 mmol) in DMF (0,3 M) was stirred at 0 °C few minutes then at

1 RT during one hour. The benzylbromide (190 mg, 1,1 mmol) was added and the reaction was stirred
2 during one hour at 60 °C and reduced. The resulting oil was dissolved in AcOEt; the organic layer was
3 washed with a saturated solution of NaHCO₃ (x2), water, saturated solution of NaCl, then dried and
4 reduced. The resulting product was purified by flash chromatography with Hexane/AcOEt=7/3 to yield a
5 beige powder (123 mg, 34%). R_f = 0.25 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 8.32-
6 8.29 (1H, d, H arom.), 8.05-8.01 (1H, m, H arom.), 7.92-7.75 (4H, m, H arom.), 7.62 (1H, s, H arom.),
7 7.50-7.45 (3H, m, H arom.), 7.40-7.20 (3H, m, H arom.), 7.07-7.03 (3H, m, H arom.), 4.86 (2H, s, -CH₂-
8 napht.), 4.78 (2H, s, -CH₂-benz.). ¹³C NMR (50 MHz, DMSO-d₆): δ 147.50, 134.74, 133.77, 133.43,
9 132.07, 130.01, 129.87, 128.72, 128.23, 128.12, 127.25, 127.18, 126.90, 126.72, 126.61, 125.23,
10 124.73, 124.38, 55.39, 55.16. Synthesis of the salt. Mp = 160 °C. HRMS m/z (M+H)⁺ 325.1699.

11 **[Benzyl-(2-naphthylmethyl)-amino]-2-methylpyridine hydrochloride (30).** Prepared from the
12 secondary amine **o** (250 mg, 1 eq), benzylbromide (200 mg, 1.1 eq) and TEA (1.2 eq) using the general
13 procedure for tertiary amines. The resulting product was purified by flash chromatography with
14 Hexane/AcOEt= 9/1, 8/2 then 7/3 to yield a yellow solid (77%). R_f = 0.15 (Hexane/AcOEt=9/1). ¹H
15 NMR (300 MHz, CDCl₃): δ 8.06-8.04 (1H, d, H arom.), 7.93-7.89 (4H, m, H arom.), 7.65-7.57 (3H, m,
16 H arom.), 7.50-7.42 (4H, m, H arom.), 7.37-7.30 (2H, m, H arom.), 7.29-7.25 (1H, m, H arom.), 7.11-
17 7.09 (1H, m, H arom.), 3.81 (2H, s, -CH₂-pyr), 3.79 (2H, s, -CH₂-napht.), 3.68 (2H, s, -CH₂-benz.). ¹³C
18 NMR (50 MHz, DMSO-d₆): δ 161.29, 142.82, 137.52, 129.59, 128.93, 127.98, 127.32, 125.79, 115.77,
19 57.66, 57.04. Synthesis of the salt. HRMS m/z (M+H)⁺ 339.1856.

20 **[Benzyl-(2-naphthylmethyl)-amino]-2-pyridine (31).** A solution of secondary amine **a** (500 mg, 1
21 eq), with BuLi (1.5 eq) in DMF (0,3 M) was stirred at 0 °C then cooled at RT during 1 h. 2-
22 Chloropyridine (253 mg, 1.1 eq) was added and the solution was stirred at RT during 1 h then at 60 °C
23 overnight and reduced. The resulting oil was dissolved in AcOEt; the organic layer was washed with a
24 saturated solution of NaHCO₃ (x2), water, saturated solution of NaCl, then dried and reduced. The
25 resulting product was purified by flash chromatography with Hexane/AcOEt=7/3 to yield an oil (32%).
26 R_f = 0.2 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 8.36-8.33 (1H, d, J = 6 Hz, H arom.),

1 8.33-7.84 (3H, m, H arom.), 7.72 (1H, s, H arom.), 7.53-7.35 (9H, m, H arom.), 6.70-6.67 (1H, dd, J1 =
2 5 Hz, J2 = 1 Hz, H arom.), 6.60-6.55 (1H, d, J = 8 Hz, H arom.), 5.04 (2H, s, -CH₂-napht.), 4.93 (2H, s,
3 -CH₂-benz.). ¹³C NMR (50 MHz, DMSO-d₆): δ 159.04, 148.43, 138.78, 137.95, 136.32, 133.87,
4 133.11, 112.78, 106.44, 78.03, 77.45, 76.82, 51.49, 51.26. Mp = 76 °C. HRMS m/z (M+H)⁺ 325.1540.

5 **[Benzyl-(2-naphthylmethyl)-amino]-4-pyrimidine hydrochloride (32).** Prepared from the
6 secondary amine **a** (350 mg, 1.3 mmol), 4-chloropyrimidine (150 mg, 1.3 mmol) and TEA (150 mg, 1.5
7 mmol) using the general procedure for tertiary amines. The resulting product was purified by flash
8 chromatography with Hexane/AcOEt=5/5 to yield a yellow solid (250 mg, 53%). R_f = 0.2
9 (Hexane/AcOEt=7/3). ¹H NMR (300 MHz, CDCl₃): δ 8.72 (1H, s, H arom.), 8.28-8.17 (1H, d, J = 6 Hz,
10 H arom.), 7.86-7.75 (4H, m, H arom.), 7.60 (2H, m, H arom.), 7.50-7.46 (3H, m, H arom.), 7.40-7.25
11 (3H, m, H arom.), 6.48-6.45 (1H, d, J = 6 Hz, H arom.), 4.95 (2H, s, -CH₂-napht.), 4.83 (2H, s, -CH₂-
12 benz.). ¹³C NMR (50 MHz, CDCl₃): δ 162.34, 158.79, 155.96, 137.10, 134.56, 133.79, 133.24, 129.19,
13 128.13, 127.96, 127.52, 126.78, 126.37, 126.09, 125.64, 103.55, 50.77, 50.57, 30.11. Synthesis of the
14 salt. HRMS m/z (M+H)⁺ 326.1652.

15

16 **Biology.**

17 **Cloning.** NK2R cDNA. Rat NK2R cDNA in 5' frame fusion with EGFP cDNA was cloned in pCEP4
18 expression vector (Invitrogen) as described.⁶

19 **Cell culture and Buffer.** HEK293 cells were stably transfected by calcium phosphate precipitation⁴
20 and selected with 500µg/ml Hygromycin B. Cells were assayed in Hepes-BSA buffer (137.5mM NaCl,
21 1.25mM MgCl₂, 1.25mM CaCl₂, 6mM KCl, 5.6mM glucose, 10mM Hepes, 0.4mM NaH₂PO₄, 1%
22 Bovine Serum Albumin (w/v), pH 7.4) supplemented with protease inhibitors.⁶

23 **FRET measurements.** Fluorescence binding. Interaction of fluorescent peptides with chimeric EGFP-
24 rNK2 was monitored on a Fluorolog 2 spectrofluorometer (SPEX) with excitation set at 470 nm
25 (bandwidth = 5nm), as a decrease of EGFP emission at 510 nm (bandwidth = 10 nm) reflecting
26 fluorescence resonance energy transfer towards the acceptor group Bodipy as described.⁴ Cell (106

1 cells/ml) or membrane suspensions (100 µg.protein/ml) in Hepes-BSA buffer were placed at 20 °C in a
2 1ml-quartz cuvette under constant stirring. Addition of fluorescent agonist to the suspension results in a
3 decrease of EGFP fluorescence at 510 nm. The intensity of the fluorescence variation is proportional to
4 receptor sites occupancy. Typically, saturation of receptor sites results in 40% decrease of total
5 preparation fluorescence. Dissociation experiments are initiated by addition of an excess of unlabelled
6 NKA (1 µM) and recorded as a recovery of EGFP fluorescence intensity as a function of time.

7 **Data analysis.** The KaleidaGraphTM software was used for fitting NKABO binding traces. Bi-
8 exponential dissociation was defined by equation: $[F] = f(t) = A1 \cdot \exp(-V1 \cdot t) + A2 \cdot \exp(-V2 \cdot t)$. Where,
9 t is the time in sec.; $[F]$ is the FRET signal accounting for ligand bound to the receptor; $V1$ and $V2$ the
10 off rate constants k_{off} of respectively rapid and slow dissociation events in sec⁻¹, $A1$ and $A2$ are the
11 amplitudes of respectively rapid and slow dissociation.

12 **Intracellular calcium determinations.** Determinations were carried out as described.^{6,30} Briefly,
13 adherent cells were loaded with 5 µM Indo-1 for 45 min at 37 °C, dissociated in PBS and suspended in
14 Hepes buffer (in mM: 137.5 NaCl, 1.25 MgCl₂, 1.25 CaCl₂, 6 KCl, 5.6 glucose, 10 Hepes, 0.4
15 NaH₂PO₄, 1% BSA (w/v), pH 7.4) in Hepes buffer (in mM: 137.5 NaCl, 1.25 MgCl₂, 1.25 CaCl₂, 6
16 KCl, 5.6 glucose, 10 Hepes, 0.4 NaH₂PO₄, 1% BSA (w/v), pH 7.4). Cell suspension measurements
17 were made at 21 °C in a 1-ml cuvette on the SPEX Fluorolog 2 spectrofluorometer with excitation set at
18 355 nm and time-based emission detected at 405 nm and 475 nm. Recordings were normalized
19 according to the maximal intensity of fluorescence detected upon addition of the detergent digitonin and
20 plotted as 475/405 ratios. Data points reported in the figure correspond to the area under the response
21 curve in arbitrary units.

22 **cAMP measurements.** Functional NK2R-mediated coupling to adenylyl cyclase was assessed by
23 measuring the dose-dependent stimulatory effects of neurokinin A on cAMP accumulation. HEK293
24 cultures expressing NK2 receptors were harvested and seeded in 24-well plates coated with collagen (60
25 mg/ml) at an initial density of 50,000. After 3 days of culture (~80% confluency), the cells were treated
26 as previously described^{6,30} The inhibitory effect of LPI805 and LPI827 on NKA-stimulated cAMP

1 accumulation was determined in the presence of 10 μ M of each compound. The reaction was stopped by
2 addition of one volume ice-cold 0.2M HCl and cAMP was determined by radioimmunoassay.

3

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6 (grant LSHB-CT-2003-503337).

7

8 SUPPORTING INFORMATION AVAILABLE: extended analytical data on the most potent
9 compounds are available free of charge via the Internet at <http://pubs.acs.org>.

10

Figure 1. Principle of the FRET-based assay exemplified with compound **1**. Association phase P1: at time $t = 50$ s, 20 nM NKA-Bo are added to a suspension of cells expressing EGFP-NK2R and association is followed as a decrease of EGFP fluorescence intensity at 510 nm. Incubation with test compound phase P2: at 450 s, the tested allosteric compound is added and equilibration is allowed to proceed for 150 s. Dissociation phase P3: at 600 s, 20 μ M of the non fluorescent ligand NKA are added and NKA-Bo dissociation is recorded. In the absence of allosteric modulator, NKA-Bo dissociates according to the blue trace; in the presence of the allosteric compound **1**, it dissociates according to the red trace.

Figure 2. Effect of compounds **1** and **16** on NKA-evoked intracellular calcium. Intracellular calcium release dose-response relationship to increasing concentrations of NKA (open circles, $EC_{50} = 0.55 \pm 0.05$ nM) were recorded. The effect of the presence of compound **1** (open squares, $EC_{50} = 0.50 \pm 0.05$ nM) or compound **16** (filled squares, $EC_{50} = 0.62 \pm 0.04$ nM) is shown. While compound **1** does not modify the sensitivity to NKA nor the amplitude of the response, compound **16** enhances the amplitude of the maximal response to $120 \pm 5\%$.

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3 **Figure 3.** Effect of compounds **1** and **16** on NKA-evoked cAMP production in HEK cells. cAMP dose-
 4 response relationship to increasing concentrations of NKA (open circles, $EC_{50} = 64.8 \pm 2.1$ nM) were
 5 recorded. The effect of the presence of 10 μ M compound **1** (open squares, $EC_{50} = 74.8 \pm 3.5$ nM) or
 6 compound **16** (filled squares, $EC_{50} = 64.8 \pm 1.4$ nM) is shown. Each data point is the mean $\pm$ s.d. of 6
 7 determinations. Compound **1** reduces the maximal amplitude of the cAMP response by $33 \pm 5\%$ and
 8 compound **16** reduces it by $47 \pm 4\%$ at the same concentration, showing improved activity over
 9 compound **1**.

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3 **Figure 4.** Two putative binding domains for NK2 receptor allosteric modulators (view from the outside
4 of the cell). The models have been built classically by homology with bovine opsin.³¹ Trp1.35, Tyr1.39
5 on the top of TM 1 and Tyr2.64 on the top of TM2 (green) form the first aromatic cluster, pointing
6 towards the entrance of the orthosteric binding cleft. Phe2.56, 2.60 (TM2) and Phe3.24, 3.27 and 3.31
7 (top of TM3), in blue, form the second aromatic cluster, pointing toward the outside of the receptor, in
8 the transmembrane region. Similar domains are obtained based on the most recent crystal structures of
9 adrenergic and adenosine receptors.³²

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Scheme 1. Synthesis of analogues of the allosteric modulator **1**.

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7 **Reagents and conditions.** i) MeOH, 60 °C ; ii) NaBH₄, RT ; iii) NEt₃, DMF, 60 °C ; iv) amine, BOP,
8 NMM, DMF, RT ; v) alcohol, BOP, NMM, DMF, RT.

9
10 **Chart 1.** Structure of **1**, [N-Benzyl,N-(2-naphthylmethyl)-amino]-acetonitrile, and pharmacomodulation
11 positions (in circles).

1

2 **Table 1.** Percentages of NK₂-R active conformations A1 and A2 with or without compound **1** in
3 presence of 20 nM NKA-Bo.

	Quick Dissociation (A1 conformation)	Slow Dissociation (A2 conformation)
Without 1	30%	70%
1 at 10 μ M	50%	50%
1 at 50 μ M	95%	5%

4

5 **Table 2.** Allosteric activity of **1** and of its benzyl-analogues, at 10 μ M in a kinetic FRET-based assay.

N°	R1	% of A1 conformation. ^a	FRET inhibition ^b	n ^c
1	Benzyl	50% $\pm$ 3	0%	10
2	4-Bromobenzyl	45% $\pm$ 1	0%	3
3	2-Bromobenzyl	43% $\pm$ 2	0%	3
4	4-Iodobenzyl	47% $\pm$ 3	0%	3
5	Phenethyl	39%	20%	1
6	4-Methoxybenzyl	54% $\pm$ 1	0%	2

6

7 ^a Percentage of A1 conformation, evaluated with a two exponential equation. ^b FRET inhibition,
8 expressed as a percentage of the reversal in FRET caused by the NKA. ^c n, number of experiments.

9

10

1 **Table 3.** Allosteric activity of naphthyl-analogues of **1**, at 10 μ M in a kinetic FRET-based assay.

N ^o	Ar	% of A1 conformation. ^a	FRET inhibition ^b	n ^c
1	Benzyl	50% $\pm$ 3	0%	10
7	3-Chlorobenzyl	45%	0%	1
8	4-Chlorobenzyl	53%	0%	1
9	(1H)Indol-3-ylmethyl	-	-	1
10	Quinolin-2-ylmethyl	47% $\pm$ 4	0%	3
11	Quinolin-3-ylmethyl	46% $\pm$ 4	21%	3
12	Cinnamyl	47% $\pm$ 3	0%	3
13	4-Methoxybenzyl	49% $\pm$ 2	0%	3
14	4-Trifluoromethylbenzyl	48% $\pm$ 3	0%	3

2
3 ^a Percentage of A1 conformation, evaluated with a two exponential equation. ^b FRET inhibition,
4 expressed as a percentage of the reversal in FRET caused by the NKA. ^c n, number of experiments.

1 **Table 4.** Allosteric activity of acetonitrile analogues of **1**, at 10 μ M in a kinetic FRET-based assay.

N°	R2	% of A1 conformation. ^a	FRET inhibition ^b	n ^c
15	Propargyl	51% $\pm$ 6	0%	3
16	Butyronitril	68% $\pm$ 1	0%	3
17	Ethyl ethanoate	51% $\pm$ 6	0%	3
18	Acetic acid	35% $\pm$ 2	0%	3
19	Ethyl pentoate	52% $\pm$ 3	0%	3
20	Pentanoic acid	44% $\pm$ 3	0%	3
21	Hexadecanoyl ethanoate	38% $\pm$ 0	0%	2
22	Phenethyl ethanoate	46% $\pm$ 2	0%	3
23	2-Hydroxyethyl ethanoate	53% $\pm$ 3	0%	3
24	Morpholinoethyl ethanoate	42% $\pm$ 2	0%	4
25	Butyl acetamide	54% $\pm$ 2	0%	3
26	Piperidino-acetamide	44%	0%	1
27	N-Methylpiperazino-acetamide	58%	0%	1
28	N-Phenylpiperazino-acetamide	43% $\pm$ 2	0%	3
29	3-Pyridine	59% $\pm$ 2	0%	2
30	2-methylpyridine	51% $\pm$ 1	24%	3
31	2-Pyridine	56% $\pm$ 4	17%	2
32	4-Pyrimidine	61% $\pm$ 3	34%	3

2 ^a Percentage of A1 conformation, evaluated with a two exponential equation. ^b FRET inhibition,
3 expressed as a percentage of the reversal in FRET caused by the NKA. ^c n, number of experiments.

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REFERENCES.

- (1) Christopoulos, A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 198-210.
- (2) May L.T.; Leach K.; Sexton P.M.; Christopoulos, A. Allosteric modulation of G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 1-51.
- (3) (a) Forster, T. Energiewanderung und fluoreszenz. *Naturwissenschaften* **1946**, *6*, 166-175. (b) Clegg, R. M. Fluorescence energy transfer. *Curr. Opin. Biotechnol.* **1995**, *6*, 103-110.
- (4) Vollmer, J.Y.; Alix, P.; Chollet, A.; Takeda, K.; Galzi, J.L. Subcellular compartmentalization of activation and desensitization of responses mediated by NK2 neurokinin receptors. *J. Biol. Chem.* **1999**, *274*, 37915-37922.
- (5) Zimmer, M. Green fluorescent protein (GFP): applications, structure and related photophysical behaviour. *Chem. Rev.* **2002**, *102*, 759-781.
- (6) Palanche, T.; Ilien, B.; Zoffmann, S.; Reck, M.P.; Bucher, B.; Edelstein, S.J.; Galzi, J.L. The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. *J. Biol. Chem.* **2001**, *276*, 34853-34861.
- (7) Ilien, B.; Franchet, C.; Bernard, P.; Morisset, S.; Weill, C.O.; Bourguignon, J.J.; Hibert, M.; Galzi, J.L. Fluorescence resonance energy transfer to probe human M1 muscarinic receptor structure and drug binding properties. *J. Neurochem.* **2003**, *85*, 768-778.
- (8) Maillet, E.L. ; Pellegrini, N. ; Valant, C. ; Bucher, B. ; Hibert, M. ; Bourguignon, J.J. ; Galzi, J.L. A novel conformation specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties, *FASEB J.* **2007**, *21*, 2124-2134.
- (9) Galzi, J.L.; Hibert, M.; Bourguignon, J.J.; Maillet, E. Method for isolating an allosteric effector of a receptor. WO Patent 03107004, December **2003**.

- 1 (10) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C.A.; Motoshima, H.; Fox, B.A.; Le Trong, I.;
2 Teller, D.C.; Okada, T.; Stenkamp, R.E.; Yamamoto, M.; Miyano, M. Crystal structure of Rhodopsin: a
3 G protein coupled receptor. *Science* **2000**, *289*, 739-745.
- 4 (11) Bhogal, N.; Donnelly, D.; Findlay, J.B. The ligand binding site of the neurokinin 2 receptor. Site-
5 directed mutagenesis and identification of neurokinin A binding residues in the human neurokinin 2
6 receptor. *J. Biol. Chem.* **1994**, *269*, 27269-27274.
- 7 (12) Labrou, N.E.; Bhogal, N.; Hurrell, C.R.; Findlay, J.B. Interaction of Met297 in the seventh
8 transmembrane segment of the tachykinin NK2 receptor with neurokinin A. *J Biol Chem.* **2001**, *276*,
9 37944-9.
- 10 (13) Milligan, G.; Smith, N.J. Allosteric modulation of heterodimeric G-protein coupled receptors.
11 *Trends Pharmacol Sci.* **2007**, *28*, 615-20.
- 12 (14) Milligan, G.; Bouvier, M. Methods to monitor the quaternary structure of G protein-coupled
13 receptors. *FEBS J.* **2005**, *272*, 2914–2925.
- 14 (15) Cherezov, V.; Rosenbaum, D. M.; Hanson, M. A.; Rasmussen, S.; Thian, F.; Kobilka, T. S.;
15 Choi, H.-J.; Kuhn, P.; Weis, W.; Kobilka, B.; Stevens, R. High-Resolution Crystal Structure of an
16 Engineered Human β 2-Adrenergic G Protein–Coupled Receptor. *Science* **2007**, *318*, 1258–1265.
- 17 (16) Warne, T.; Maria, J.; Serrano-Vega, M.; Baker, J.; Moukhametzianov, R; Edwards, P.;
18 Henderson, R.; Leslie, A.; Tate, C.; Schertler, G. Structure of a β 1-adrenergic G-protein-coupled
19 receptor. *Nature* **2008**, *454*, 486-491.
- 20 (17) Veli-Pekka Jaakola, V.; Griffith, M.; Hanson, M.; Cherezov, V.; Chien, E.; Lane, R.; IJzerman,
21 A.; Stevens, R. The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an
22 Antagonist. *Science* **2008**, *322*, 1211-1217.

- 1 (18) Springael, J.-Y.; Urizar, E.; Costagliola, S.; Vassart, G.; Parmentier, M. Allosteric properties of G
2 protein coupled receptor oligomers. *Pharmaco. Ther.* **2007**, *115*, 410-418.
- 3 (19) Adham, N.; Ellerbrock, B.; Hartig, P.; Weinshank, R. L.; Branchek, T. Receptor reserve masks
4 partial agonist activity of drugs in a cloned rat 5-hydroxytryptamine_{1B} receptor expression system. *Mol.*
5 *Pharmacol.* **1993**, *43*, 427-433.
- 6 (20) Whaley, B. S.; Yuan, N.; Birnbaumer, L.; Clark, R. B.; Barber, R. Differential expression of the
7 beta-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation.
8 *Mol. Pharmacol.* **1994**, *45*, 481-489.
- 9 (21) Soudijn, W.; van Wijngaarden, I.; IJzerman, A. Allosteric modulation of G protein coupled
10 receptors: perspectives and recent developments. *Drug Discovery Today* **2004**, *9*, 752-758.
- 11 (22) Leach, K.; Sexton, P. M.; Christopoulos, A. Allosteric GPCR modulators: taking advantage of
12 permissive receptor pharmacology. *Trend Pharmacol. Sci.* **2007**, *28*, 382-389.
- 13 (23) Schwartz, T.; Holst, B. Allosteric enhancers, allosteric agonists and ago-allosteric modulators:
14 where do they bind and how do they act? *Trends Pharmacol. Sci.* **2007**, *28*, 367-373.
- 15 (24) Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. International Union of
16 Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on
17 terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **2003**, *55*, 597-606.
- 18 (25) Urban, J. D.; Clarke, W. P.; von Zastrow, M.; Nichols, D.E.; Kobilka, B.; Weinstein, H.; Javitch,
19 J. A.; Roth, B. L.; Christopoulos, A.; Sexton, P. M.; Miller, K. J.; Spedding, M.; Mailman, R. B.
20 Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.*
21 **2007**, *320*, 1-13.
- 22 (26) Kenakin, T. Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends*
23 *Pharmacol. Sci.* **1995**, *16*, 232-238.

- 1 (27) Hermans, E. Biochemical and pharmacological control of the multiplicity of coupling of G
2 protein coupled receptors. *Pharmacol. Ther.* **2003**, *99*, 25-44.
- 3 (28) Tateyama, M.; Kubo, Y. Dual signalling is differentially affected by different active states of the
4 metabotropic glutamate receptor 1 α . *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 1124-1128.
- 5 (29) Aurelio, L.; Valant, C.; Flynn, B.; Sexton, P.; Christopoulos, A. ; Scammells, P. Allosteric
6 modulators of the adenosine A1 receptor : synthesis and pharmacological evaluation of 4 substituted 2-
7 amino-3-benzoylthiophenes. *J. Med. Chem.* **2009**, *52*, 4543–4547.
- 8 (30) Lecat, S., Bucher, B., Mely, Y. and Galzi, J.L. Mutations in the extracellular amino-terminal
9 domain of the NK2 neurokinin receptor abolish cAMP signaling but preserve intracellular calcium
10 responses. *J. Biol. Chem.* **2002**, *277*, 42034-42048.
- 11 (31) K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima and B.A. Fox *et al.*, Crystal
12 structure of rhodopsin: a G protein-coupled receptor. *Science* **2000**, *289*, 739–745.
- 13 (32) Topiol, S.; Sabioa, M. X-ray structure breakthroughs in the GPCR transmembrane region.
14 *Biochem. Pharmacol.* **2009**, *38*, 11-20.

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