Design, Synthesis and Biological Studies of a Library of NK1-Receptor LigandsBased on a 5-arylthiosubstituted 2-amino-4,6-diaryl-3-cyano-4*H*-pyran Core: Switch from Antagonist to Agonist Effect by Chemical Modification

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ABSTRACT. A library of 5-arylthiosubstituted 2-amino-4,6-diaryl-3-cyano-4*H*-pyrans has been synthesized as a new family of non-peptide NK1 receptor ligands by a one-pot cascade

process. Their biological effects via interaction with the NK1 receptor were experimentally determined as percentage of inhibition (for antagonists) and percentage of activation (for agonists), compared to the substance P (SP) effect, in IPone assay. A set of these amino compounds was found to inhibit the action of SP, and therefore can be considered as a new family of SP-antagonists. Interestingly, the acylation of the 2-amino position causes a switch from antagonist to agonist activity. The 5-phenylsulfonyl-2-amino derivative **17** showed the highest antagonist activity, while the 5-*p*-tolylsulfenyl-2-trifluoroacetamide derivative **20***R* showed the highest agonist effect, both containing a *p*-nitrophenyl group at the 4 position of the pyran ring. As expected, in the case of the 5-sulfinylderivatives, there was an enantiomeric discrimination in favor of one of the two enantiomers, specifically those with (S_S,R_C) configuration. The anticancer activity studies assessed by using human A-549 lung cancer cells and MRC-5 non-malignant lung fibroblasts, revealed a statistically significant selective cytotoxic effect of some of these 2-amino-4*H*-pyran derivatives toward the lung cancer cells.These studies demonstrated that the newly synthesized 4*H*-pyran derivatives can be used as a starting point for the synthesis of novel SP-antagonists with higher anticancer activity in the future.

1. Introduction

Substance P (SP, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), Figure 1, neurokinin A (NKA), and neurokinin B (NKB) are all mammalian tachykinins acting as both neurotransmitters and neuromodulators.[1] These peptides exert their biochemical effects in the central nervous system (CNS) and in peripheral tissues through their binding to G protein-coupled receptors NK1, NK2, and NK3, respectively.[2] These tachykinins are involved in pain transmission, inflammation and smooth muscle contraction, vasodilatation, gland secretion, and activation of

the immune system.[3] Moreover, NK1 receptor (NK1R) is present in brain regions involved in the regulation of affective behaviour and the mediation of stress anxiety and depression.[3,4] Importantly, it has also been reported that the NK1R is highly over-expressed in a large number of aggressive tumours,[5]particularly glioma, astrocytomas and glioblastomas,[6] where the level of expression is correlated with the degree of malignancy.[7] Indeed, the rapid internalization of SP inside the cells upon interaction with NK1R,[8] associated with the overexpression of NK1R in most cancer cells, allowed the development of an efficient receptor-mediated delivery system of synthetic antibodies into tumour cells.[9] The observation that the release of SP is associated with several psychopathological processes makes NK1R a therapeutic target of great relevance. Consequently, NK1R antagonists are potential therapeutic agents for pathologies such as migraine,[4a] rheumatoid arthritis,[4b] asthma, inflammatory bowel disease and the regulation of central nervous system disorders such as Parkinson's disease, anxiety or depression, emesis,[4c,d] as well as cancer treatment.[10]

The first NK1R antagonist designs, which were based on SP structure, afforded peptide antagonists with very low affinities and limited metabolic stability.[11] The discovery at the beginning of the 1990s of the first non-peptide antagonist, CP-96345,[12] boosted the research in this area not only in academia but also in industry with almost all important pharmaceutical companies investing in this field with the aim to identify selective and potent NK1R antagonists.[13] More than two decades of immense synthetic and economic efforts have allowed the discovery of a large number of structurally diverse NK1R antagonists, though none of them with the desired therapeutic success. Currently, there are only two NK1R antagonist-based drugs in the market, Aprepitant (Merck) and its water soluble injectable form, Fosaprepitantdimeglumine (Merck), approved for prevention of chemotherapy-induced nausea

and vomiting[14] (Figure 1), and the recently launched Rolapitant (Tesaro) approved for the prevention of delayed nausea and vomiting associated with initial and repeat courses of emetogenic cancer chemotherapy.[15]

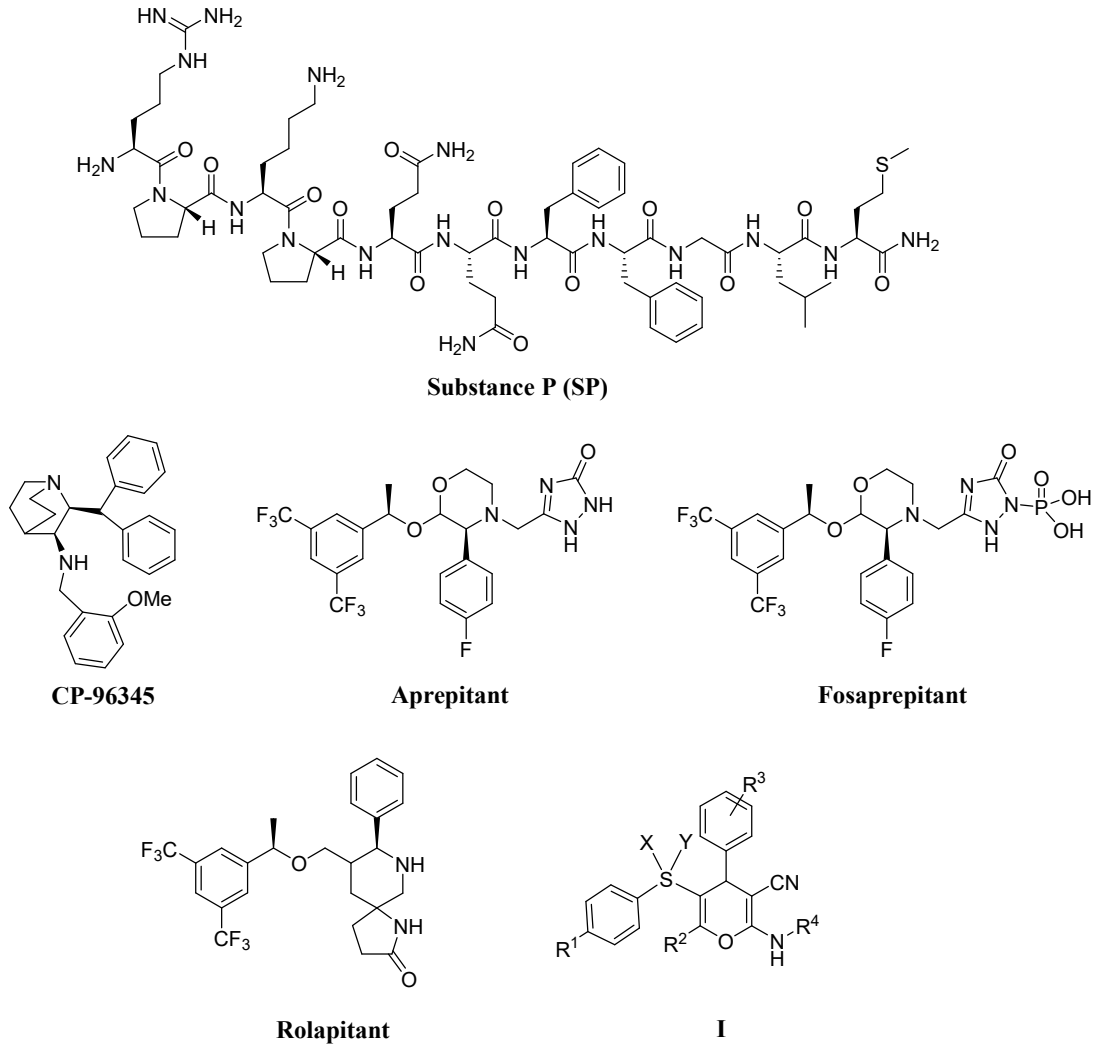


Figure 1. Structure of substance P (SP), thenonpeptide NK1R antagonists:CP-96345, Aprepitant, Fosaprepitant and Rolapitant, and the 5-arylthio-2-amino-4*H*-pyrans (**I**) prepared for this study.

It must be taken into account that the exact structure of the NK1R, which belongs to the superfamily of G protein-coupled protein receptors (GPCRs),[16] is still missing in the literature. As integral membrane proteins, the structural study of GPCRs through X-ray diffraction analysis remained elusive for decades.[17]Indeed, even though there are more than 800 GPCRs, only the structure of 35 of them is known.[18] The first GPCR structure was determined in 2000[17] but it was not until 2007 when the other 34 structuresstarted to be elucidated, the crystal structure of the ETB receptor being the most recently determined GPCR.[19]

The design and synthesis of new non-peptide molecules with high affinity for NK1Rs, and preferentially with a different chemical structure from known NK1R antagonists, is an important area in modern medicinal chemistry. To achieve this goal, in addition to intuition, traditional drug design approaches are applied, along with combinatorial chemistry and computer aided design. The considerable number of structurally diverse nonpeptidicNK1R antagonists found, allowed the proposition of a preliminary pharmacophore model, Figure 2.[20]The pharmacophore consists of at least two aromatic rings kept in a fixed orientation by various scaffolds, containing not less than one hydrogen-bond acceptor.[21]

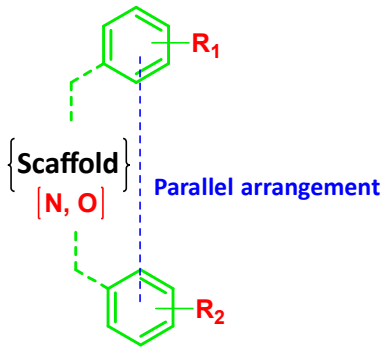


Figure 2. Generalized nonpeptide NK1R antagonist pharmacophore consisting of two (or more) aromatic rings held together by various scaffolds, which contains at least one hydrogen-bond acceptor.

The relative disposition of the aromatics in such ligands has also been widely studied and has revealed the hypothesis for the receptor bound conformation of NK1R antagonists. In most cases, the fixed orientation between the two aromatic groups aforementioned can be either a parallel face-to-face,[22] or perpendicular "T" or edge-on "L" arrangement.[23]

On the other hand, it is worth noting that the discovery of non-peptide tachykinin agonists can also be of therapeutic relevance in the treatment of some diseases, as the angiogenesis-dependent diseases where endothelial cell proliferation is required to promote vascularization and healing of ischemic or damaged tissues.[24]However, in contrast to the number of non-peptide NK1 receptor antagonists described so far, the agonists for these receptors still appears to be mostly confined to peptide compounds. Some studies have shown that slight structural variations of certain antagonists produce marked changes in their activities and, in some cases, it causes the intrinsic efficacy to shift towards an agonistic activity, accompanied by only minor variations in binding affinity.[25] Despite being aware that this behaviour is only confined to certain compounds and the structural requirements that define an agonist versus an antagonist are poorly understood, it is worth considering the possibility of interconverting GPCR antagonists to agonist by simple structural modifications.

Based on these premises, and in connection with our interest in the synthesis of optically pure sulfinylderivatives with biological and pharmacological significance,[26] herein we present our studies on the design and synthesis of 5-arylthiosubstituted 2-amino-4,6-diaryl-3-cyano-4*H*-pyransas new NK1R antagonists, and the quantification of their abilities to inhibit the synthesis

of inositol 1-phosphate (IP_1), by Homogeneous Time-Resolved Fluorescence (HTRF) technology using the IPone test. Additionally, we have evaluated the possibility of employing small structural changes to interconvert the antagonists to agonists as part of viable GPCR drug discovery strategy. On the other hand, we have also evaluated the capacity of these 5-arylthio-2-amino-4,6-diaryl-3-cyano-4*H*-pyran derivatives to actas antitumoral agents towards cancer cell lines such as the human lung adenocarcinoma.

2. Results and discussion

2.1. Design

To generate the new NK1 antagonist, we utilized a ligand-based design methodology. The basis of the present study is the observation that despite their structural disparity, CP-96345 and compound **1**(S_S,R_C) possess significant common features in the solid state, as shown by their X-ray crystal structures (Figure 3). CP-96345 is a quinuclidinic compound with two stereogenic carbon atoms, while compound **1**(S_S,R_C) belongs to the 2-amino-4*H*-pyran family, and encloses a stereogenic carbon atom vicinal to a chiral sulfoxide moiety. As illustrated in Figure 3, both compounds have two parallel aromatic rings, stabilized by a π–π stacking interaction, and at least one hydrogen bonding acceptor atom. More specifically, compound **1**(S_S,R_C) meets the requirements of the advanced NK1R antagonists pharmacophore proposed by Klebe's virtual NK1R model.[21,27]

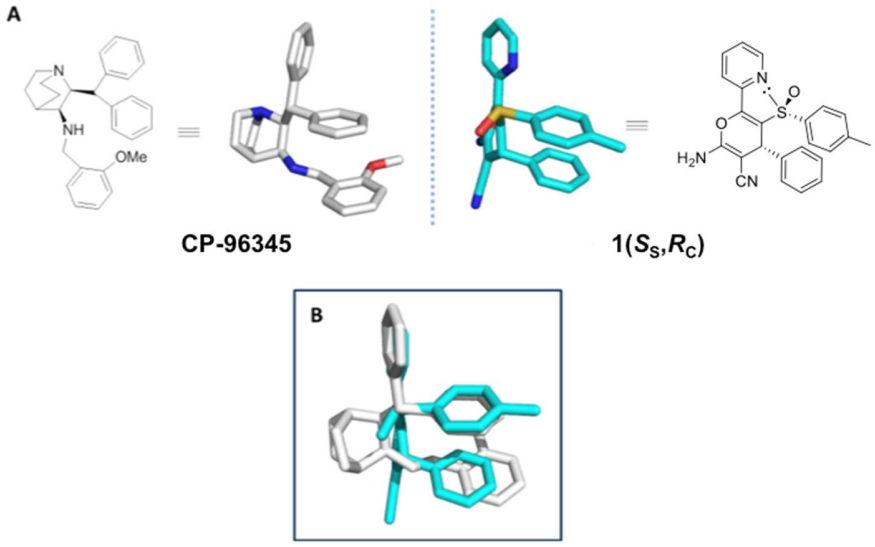


Figure 3. A) X-Ray crystal structures of CP-96345 (left),[28] and 5-*p*-tolylsulfinyl-2-amino-4*H*-pyran derivative**1**(S_S,R_C) (right). B) Superposed view of CP-96345 and**1**(S_S,R_C).

NK1R Klebe's virtual model,[21] is a ligand-supported homology model based on the known crystal structure of the rhodopsin receptor and on the structure of CP-96345. The quality of the model was validated by checking its ability to accommodate additional known NK1 antagonists from structurally diverse classes, leading to the development of an advanced pharmacophore model. We have observed that in the modeled NK1R/CP-96345 complex, compound **1**(S_S,R_C) could replace CP-96345 and maintain all the hydrophobic and hydrogen bond interactions proposed for the reference antagonist with the amino acids on the active site of the receptor, namely with glutamine 165, glutamic acid 193, histidine 197, isoleucine 204 and histidine 265 (Figure 4).

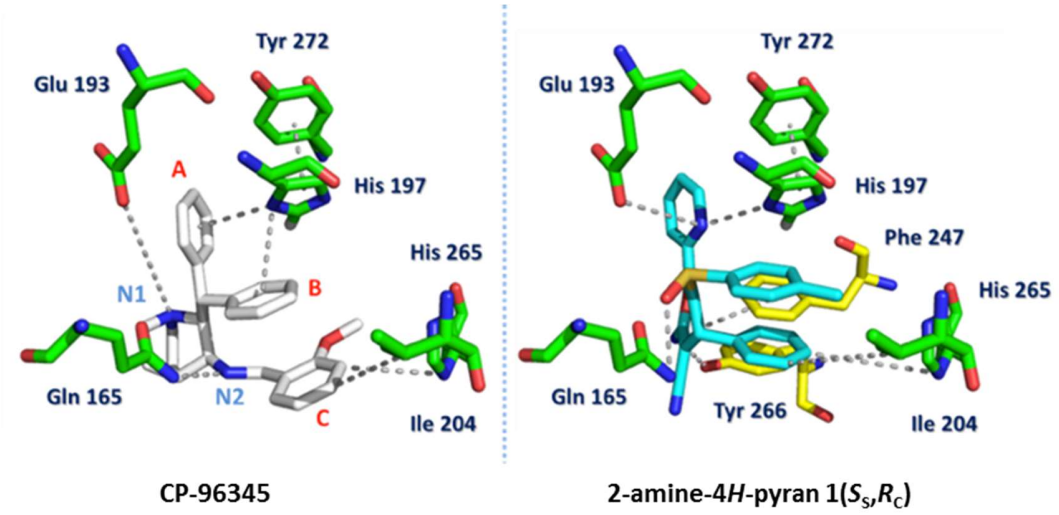


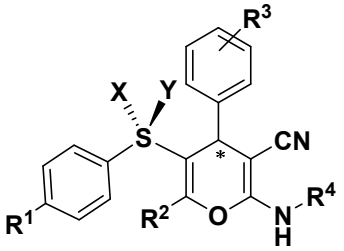
Figure 4. Modeled complex of the NK1R with CP-96345 (left) and compound **1**(S_S,R_C) (right).

Accordingly, mutation studies have shown that the most relevant interaction of the NK1R / antagonist complex is the hydrogen bond between the terminal NH_2 of the glutamine (Gln 165) and a hydrogen bond acceptor atom of the antagonist.[29] Compound **1**(S_S,R_C) positioned in the Klebe's model has the sulfinyl oxygen at 2.95 Å from Gln165, thus a hydrogen bond could be established with this amino acid. Another important interaction described for the NK1R/CP-96345 complex is the amino-aromatic hydrogen bond between the benzhydryl group of CP-96345 with His197[30], which itself is kept in place by an aromatic-aromatic interaction with Tyr272.[31]In the case of NK1R/**1**(S_S,R_C) complex, a similar interaction could be established between His197 and the pyridine ring either as an amine-aromatic interaction or as a hydrogen bond with the pyridine nitrogen that liesat 2.17 Å. Finally, an in-depth examination of the NK1R/**1**(S_S,R_C) complex shows that the amino acids Phe247 and Tyr266 are appropriately arranged to establish an effective interaction with the ligand**1**(S_S,R_C). Particularly relevant could be the aromatic-amine interaction between Phe247 and the amino group at the 3 position on the pyran ring, as well asa hydrogen bond interaction between the aforesaid amino group and the hydroxyl group of Tyr266 (2.04 Å). In summary, the former qualitative study showed a good

match between the non-peptidicantagonist CP-96345/NK1R complex and the new ligands-NK1R interactions, and indicated that the 5-arylsulfinyl-2-amino-4*H*-pyrans could have NK1R binding capacity. Therefore, taking into account these observations andthe influence of the substitution pattern ofboth phenyl rings in the pharmacophore on binding affinity,[21] we designed a batch of 5-arylthio-2-amino-4*H*-pyran compounds in order to evaluate their potential binding abilities to NK1R. This library was designed to be readilychemically accessible, and the 4*H*-pyran derivatives were specifically chosen in order to determine the influence of (*i*) the nature of the aromatic rings at C-4 and C-6 of the pyran ring, (*ii*) the oxidation state of sulphur at C-5 and the nature of its substituent, and (*iii*) the nature of the nitrogen function in position 2 (Table 1). Additionally, in view of the known low binding affinity of the (2*R*,3*S*) enantiomer(distomer) of CP-96345 for the NK1 receptor (IC_{50} = 81000 ± 12000 nM) compared to the binding affinity of the (2*S*,3*R*) enantiomer(eutomer) (IC_{50} = 3.4 ± 0.8 nM),[12] the influence of the stereochemistry of the stereogenic centres, at C4 and sulfur in the case of the sulfoxides were also analyzed.

Finally, it must be pointed out that these new drug candidates were designed taking into account the Lipinski's rules, or rules of 5, so that most of them comply with these rule of 5 guidelines (see Table 1 in Supporting Information) and are most likely to be cell permeable and orally bioavailable.[32]

Table 1. The 5-arylthio-2-amino-4,6-diaryl-3-cyano-4*H*-pyran derivatives synthesized.



X, Y= :, O
R^1= CH_3, NO_2, H,
R^2= 2-Py, 2-Furyl, 3,5-$(CF_3)_2C_6H_3$
R^3= 4-CH_3, H, 4-Cl, 4-F, 4-NO_2, 4-OMe, 3,5-$(CF_3)_2$
R^4= H, Ac, $COCF_3$

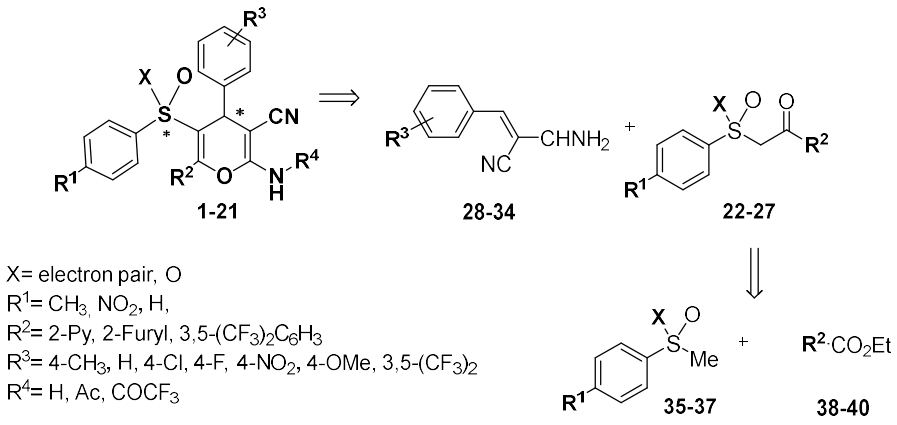
Entry	Comp.	X	Y	R^1	R^2	R^3	R^4	Conf. at C4
1	*rac*-**1**	*rac*		CH_3	2-Py	H	H	*rac*
2	**1**(S_S,R_C)	O	..	CH_3	2-Py	H	H	*R*
3	**1**(R_S,S_C)	..	O	CH_3	2-Py	H	H	*S*
4	*rac*-**2**	*rac*		CH_3	2-Py	4-CH_3	H	*rac*
5	**2**(S_S,R_C)	O	..	CH_3	2-Py	4-CH_3	H	*R*
6	**2**(R_S,S_C)	..	O	CH_3	2-Py	4-CH_3	H	*S*
7	*rac*-**3**	*rac*		CH_3	2-Py	4-Cl	H	*rac*
8	**3**(S_S,R_C)	O	..	CH_3	2-Py	4-Cl	H	*R*
9	**3**(R_S,S_C)	..	O	CH_3	2-Py	4-Cl	H	*S*
10	*rac*-**4**	*rac*		CH_3	2-Py	4-F	H	*rac*
11	**4**(S_S,R_C)	O	..	CH_3	2-Py	4-F	H	*R*
12	**4**(R_S,S_C)	..	O	CH_3	2-Py	4-F	H	*S*
13	*rac*-**5**	*rac*		CH_3	2-Py	4-NO_2	H	*rac*
14	**5**(S_S,R_C)	O	..	CH_3	2-Py	4-NO_2	H	*R*
15	**5**(R_S,S_C)	..	O	CH_3	2-Py	4-NO_2	H	*S*
16	**6**(S_S,R_C)	O	..	CH_3	2-Py	4-OCH_3	H	*R*
17	**6**(R_S,S_C)	..	O	CH_3	2-Py	4-OCH_3	H	*S*
18	**7**(S_S,R_C)	O	..	CH_3	2-Py	3,5-$(CF_3)_2$	H	*R*
19	*rac*-**8**	*rac*		CH_3	2-Furyl	4-Cl	H	*rac*
20	*rac*-**9**	*rac*		CH_3	3,5$(CF_3)_2$ C_6H_3-	4-CH_3	H	*rac*
21	*rac*-**10**	*rac*		NO_2	2-Py	4-CH_3	H	*rac*
22	*rac*-**11**	*rac*		NO_2	2-Py	4-Cl	H	*rac*
23	*rac*-**12**	*rac*		NO_2	2-Py	4-NO_2	H	*rac*
24	*rac*-**13**	*rac*		NO_2	2-Py	H	H	*rac*
25	*rac*-**14**	*rac*		H	2-Py	4-OCH_3	H	*rac*

26	*rac*-**15**	*rac*		H	2-Py	H	H	*rac*
27	*rac*-**16**	*rac*		H	2-Py	4-NO_2	H	*rac*
28	*rac*-**17**	O	O	H	2-Py	4-NO_2	H	*rac*
20	**18**(S_S,R_C)	O	..	CH_3	2-Py	4-CH_3	Ac	*R*
30	**18**(R_S,S_C)	..	O	CH_3	2-Py	4-CH_3	Ac	*S*
31	*rac*-**19**	..	..	CH_3	2-Py	4-CH_3	-$COCF_3$	*rac*
32	**20***R*	..	..	CH_3	2-Py	4-NO_2	-$COCF_3$	*R*
33	**21***R*	..	..	CH_3	2-Py	H	-$COCF_3$	*R*

2.2. Synthesis

As indicated in the retrosynthetic scheme (Scheme 1), the 5-arylthio-2-amino-4*H*-pyran derivatives can be synthesized in a *one pot* process, by reaction of the adequate β-ketothioderivative with different arylidenemalonodinitriles.[33]

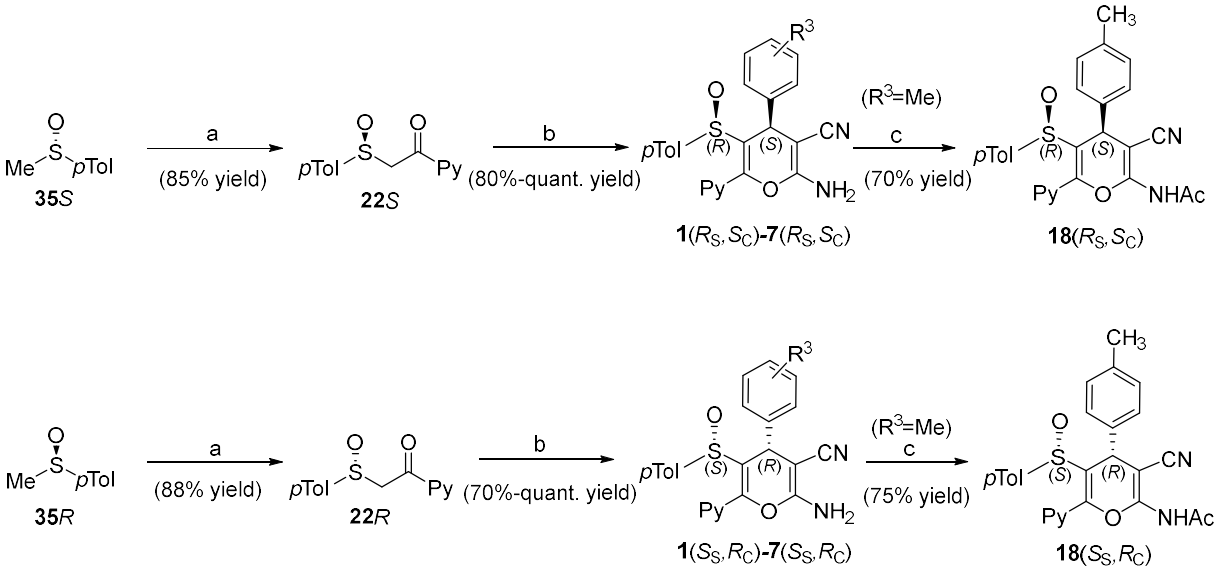
Scheme 1. Retrosynthetic scheme of 5-arylthioderivatives of 2-amino-4*H*-pyrans.



The series of the 6-pyridyl substituted sulfoxides, **1-7** (X= lone pair, R^2=Py, R^4=H, Scheme 1), was prepared in both, racemic and optically pure forms, in orderto determine the influence of the stereochemistry at sulfur and C-4 on their antagonistic effect. Both enantiomers of the

sulfinylderivatives **1-7** were obtained in enantiopure pure forms, starting from enantiopure *R* or *S* aryl methyl sulfoxides **35***R* or **35***S,* as indicated in Scheme 2. β-ketosulfoxides **22***R* or **22***S* were prepared according to the literature, by condensation of the corresponding *R* or *S* methyl sulfoxides and ethyl 2-pyridylcarboxylate **38** in the presence of lithium diisopropylamide (Scheme 2).

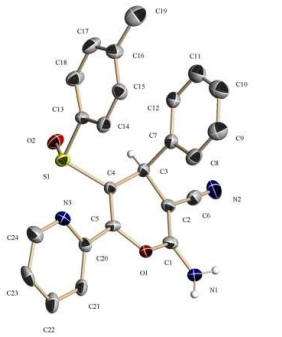
Scheme 2. Synthesis of optically pure 6-pyridyl-5-*p*-tolylsulfinyl-4*H*-pyran derivatives (**1**-**7**).[a]



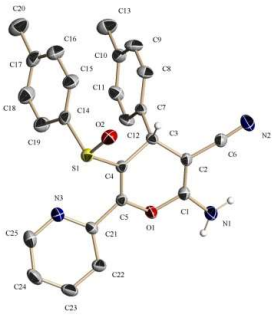
[a]Reagents and conditions: (a) 1.LDA (2 eq.), 2.ethyl 2-pyridylcarboxylate **38**, 85%; (b) arylidenemalonodinitriles **28**-**34**, piperidine (cat.), ether, (80%-quant.); (c) Ac_2O, DMAP (cat.), pyridine, 0ºC (70%)

The coupling of the arylidenemalononitriles **28-34** with the enantiopure β-ketosulfoxides **22***S*or**22***R*, using equimolar quantities of both reagents in diethyl ether with catalytic piperidine as mild base, at room temperature, led the desired compounds in good to excellent yields, as an exclusive enantiomer (Scheme 2). The reaction takes place in an addition-cyclization process,

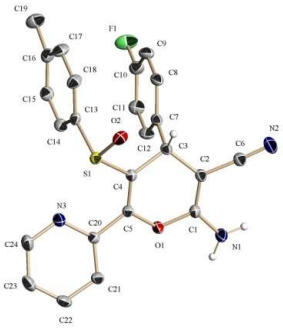
where the first addition step is an equilibrium process and it is at this moment when the new stereogenic center is formed.[34]The product formed after cyclization precipitates from the reaction medium andis isolated as a white solid by simple filtration. The nature of the solvent appears to be very important in this reaction. In general, when other solvents, such as methanol or methylene chloride were used, yields decreased dramatically. In these conditions someby-products such as the non-cyclic Michael adduct were obtained, or the reaction was not completely diastereoselective and, in some cases, the 4*H*-pyran derivative did not precipitate. The obtained compounds were in general highly crystalline, and we succeeded in obtaining single crystals of some of them for the determination of their structures (see Figure 5), which confirmed the configurational assignment of the new stereogenic carbons.



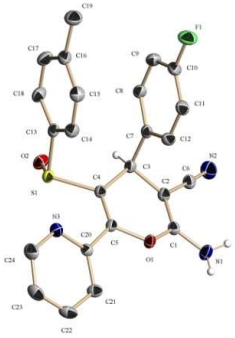
1(R_S,S_C)



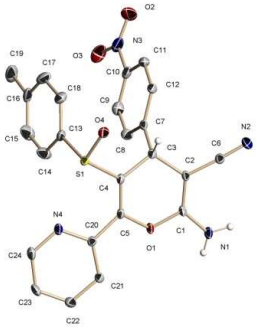
2(S_S,R_C)



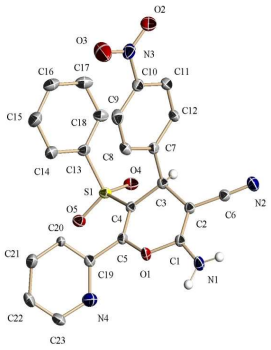
4(S_S,R_C)



4(R_S,S_C)



5(S_S,R_C)



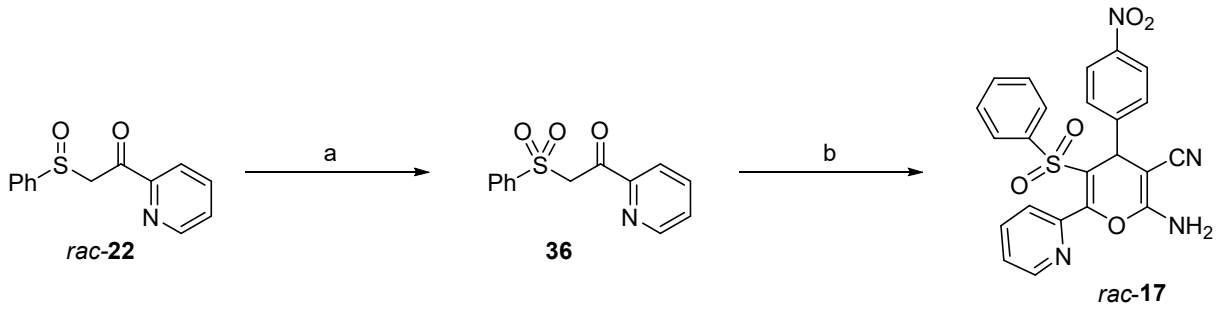
rac-**17**

Figure 5. ORTEP drawing of optically pure sulfinyl pyran derivatives **1**(R_S,S_C), **2**(S_S,R_C),**4**(S_S,R_C), **4**(R_S,S_C),**5**(S_S,R_C) and sulfone *rac*-**17**. Thermal ellipsoids are shown at the 50% probability level. The hydrogen atoms are omitted for clarity.

The X-ray analysis of the sulfinyl compounds collected in Figure 5, shows a 1,3-parallel arrangement between the aromatic rings of the two stereogenic centers (sulfinyl sulfur and carbon 4), which can be attributed to the existence of an electronic donor-acceptor interaction between both aromatic rings (i.e., a π–π stacking interaction). This favored disposition of the aromatic rings, due to the π–π stacking effects, could explain the formation of a single compound as the most stable diastereoisomer (thermodynamic control).

Treatment of **2**(S_S,R_C) and **2**(R_S,S_C) with acetic anhydride in the presence of catalytic amounts of DMAP, using pyridine as base at 0°C, yielded the corresponding sulfinyl acetamides **18**(S_S,R_C) and **18**(R_S,S_C), respectively, with good chemical yields and in enantiopure form, after chromatographic purification.The racemic (R_S,S_C/S_S,R_C) sulfinyl derivatives, (*rac*-**1**-*rac*-**16**), were prepared in a similar way by reaction of the racemic β-ketosulfoxides *rac*-**22**-*rac*-**26**, with the Michael acceptors **28**-**33**(Scheme 1). The reaction of the β-ketosulfone **27,** obtained by oxidation of the racemic sulfoxide*rac*-**25,** with the Michael acceptor **32**, under the same mild experimental conditions, gave the sulfonyl pyran derivative *rac*-**17** (Scheme 3).

Scheme 3. Synthesis of the 5-sulfonyl-4*H*-pyran derivative *rac*-**17**[a].

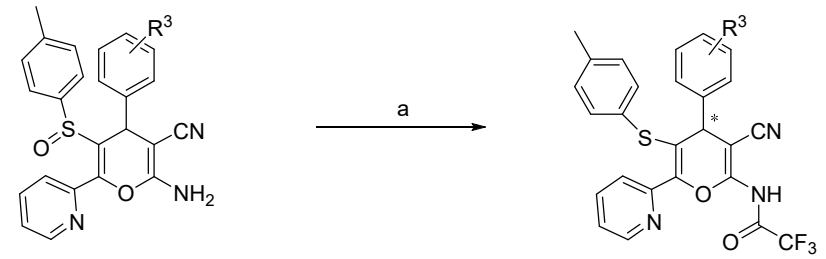


rac-**22** **36** *rac*-**17**

[a]Reagents and conditions: (a) m-CPBA, chloroform, 0ºC (96%); (b *p*-nitrobenzylidenemalononitrile**32**, piperidine (cat.), ethanol, (35%)

The influence of the nature of the solvent again proved to be crucial for the synthesis of the sulfonyl derivative, ethanol being the best solvent in this case, but the yield was only moderate. Finally, various racemic and enantiopure sulfenylderivatives were prepared by reduction of the corresponding sulfoxides, by treatment with trifluoroacetic anhydride and sodium iodide in acetone at -40°C. As expected,the trifluoroacetylation of the amino group took place concomitantly with the reduction of the sulfinyl group (Scheme 4).

Scheme 4. Synthesis of 4-aryl-5-p-tolylsulfenyl-2-trifluoroacetamide derivatives[a].



R^3= Me, *rac*-**1**
R^3= H, **2**(S_S,R_C)
R^3= NO_2, **5**(S_S,R_C)

R^3= Me, *rac*-**19**
R^3= H, **21***R*
R^3= NO_2, **20***R*

[a]Reagents and conditions: (a) 1. NaI, acetone, 2. Tf_2O, -40ºC (60-75%)

2.3. Antagonist and agonist effect evaluation.

The biological activity of the compounds was checked by their ability to inhibit or activate NK1R using the IPone assay[35]which by Homogeneous Time Resolved Fluorescence(HTRF) technology quantifies the inositol IP_1 accumulated inside the cell. Accumulation of IP_1 is an indicator of NK1R activation, so that agonist ligands of NK1R cause an increase in IP_1 levels in the absence of SP while, at the contrary, antagonist ligands produce a decrease in these levels in the presence of SP. Table 2 summarizes the results obtained with this test for the 2-amino-4*H*-pyran library, using SP and CP-96345 as agonist and antagonist controls respectively. As negative control, cells were also stimulated with DMSO in the absence of the SP and ligands (Base, Table 2).

Table2. Inactivation of IP_1 accumulation (pmoles) in NK1 receptor transfected CHO cells by 2-amino-4*H*-pyrans ($5x10^{-5}$M) and CP-96345 (10^{-7}M) in the presence of SP (8nM).

Entry	Compound	Mean (pmoles IP_1)[a,b]	±SD[b, c]	pvalue[b, d]	Mean % inhibition [b]	±SD[b, e]
1	**SP**	35.91	4.61	--	--	--
2	Base	6.52 (5.51)	2.47 (1.24)	<0.0001	--	--
3	**CP-96345**	5.29	1.52	<0.0001	85.26	2.44
4	*rac*-**1**	27.13 (23.34)	4.91 (12.57)	<0.0001 (0.0178)	24.45 (35.02)	6.84 (20.21)
5	**1**(S_S,R_C)	17.55 (20.84)	3.38 (8.87)	<0.0001 (0.0028)	51.14 (41.98)	5.43 (14.26)
6	**1**(R_S,S_C)	28.41 (25.57)	0.85 (11.38)	<0.0001 (0.0374)	20.88 (28.81)	1.34 (18.29)
7	*rac*-**2**	24.35 (24.14)	7.06 (9.18)	<0.0001 (0.0082)	32.20 (32.77)	8.79 (12.78)
8	**2**(S_S,R_C)	17.67 (22.63)	1.68 (11.60)	<0.0001 (0.0072)	50.79 (36.97)	2.79 (16.16)
9	**2**(R_S,S_C)	28.04 (22.46)	5.25 (10.35)	0.0124 (0.008)	23.74 (37.45)	7.31 (16.64)
10	*rac*-**3**	30.23 (30.9)	2.71 (12.14)	0.2017 (0.2937)	15.81 (13.95)	4.36 (19.52)
11	**3**(S_S,R_C)	28.38	3.46	<0.0001	20.98	5.56

					(30.03)	(8.13)
12	**3**(R_S,S_C)	33.83	5.78	0.149	5.80	9,29
13	*rac*-**4**	35.01	11.89	<0.0001	2.51	19.1
14	**4**(S_S,R_C)	28.37	0.87	<0.0001	20.99	0.12
15	**4**(R_S,S_C)	31.89	4.39	0.1493	11.20	6.11
16	*rac*-**5**	25.54 (25.12)	5.07 (5.06)	<0.0001 (0.0126)	28.88 (30.04)	3.53 (8.13)
17	**5**(S_S,R_C)	18.07 (20.37)	1.85 (6.36)	<0.0001 (0.0013)	49.67 (43.29)	2.57 (10.22)
18	**5**(R_S,S_C)	28.5 (20.27)	7.65 (5.10)	<0.0001 (0.1863)	20.63 (18.50)	10.65 (20.22)
19	**6**(S_S,R_C)	20.15 (23.52)	1.40 (9.10)	0.0002 (0.0058)	43.89 (34.51)	2.25 (12.67)
20	**6**(R_S,S_C)	25.82 (37.50)	9.72 (3.94)	<0.0001 (0.6833)	28.11(-4.43)	13.53 (6.34)
21	**7**(S_S,R_C)	25.64 (25.14)	13.52 (10.85)	0.0038 (0.0289)	28.61 (30.04)	21.73 (8.13)
22	*rac*-**8**	31.41	3.68	0.1441	12.52	5.92
23	*rac*-**9**	31.78	3.38	0.0034	11.51	5.43
24	*rac*-**10**	22.22	2.26	<0.0001	38.12	3.63
25	*rac*-**11**	24.36	2.81	0.0013	32.18	4.51
26	*rac*-**12**	15.77 (23.49)	0.58 (11.80)	<0.0001 (0.0166)	56.08 (35.58)	0.93 (18.97)
27	*rac*-**13**	15.57	0.68	<0.0001	56.63	1.10
28	*rac*-**14**	31.92	1.67	0.1738	11.10	2.69
29	*rac*-**15**	28.81 (24.43)	6.12 (7.83)	0.0345 (0.013)	19.77 (31.97)	6.97 (12.59)
30	*rac*-**16**	19.43 (23.89)	3.43 (2.28)	<0.0001 (0.0175)	45.89 (33.47)	5.51 (4.50)
31	*rac*-**17**	11.31 (19.56)	1.97 (7.75)	<0.0001 (0.0002)	68.51 (45.52)	2.46 (9.65)
32	**18**(S_S,R_C)	18.95 (23.94)	3.01 (8.59)	0.0006 (0.0064)	47.22 (33.32)	4.19 (11.96)
33	**18**(R_S,S_C)	22.60 (28.24)	4.63 (11.64)	<0.0001 (0.1126)	37.08 (21.36)	6.45 (18.72)
34	*rac*-**19**	22.24 (34.56)	4.41 (3.82)	<0.0001 (0.6112)	38.07 (3.77)	5.50 (4.34)
35	**20***R*	27.36	1.72	0.0087	23.82	2.77
36	**21***R*	22.68 (33.42)	2.26 (10.49)	<0.0001 (0.5727)	36.84 (6.93)	3.15 (16.86)

[a]The IP_1 accumulation was measured as described in the experimental section.[b]Corresponding values for 10^{-5}M concentration in brackets.[c]Data are means ±SD of IP_1 concentration (pmol) and illustrated from a representative experiment performed at least three times.[d]For each compound, a statistical analysis between control and treated cells was done.[e]Data are means ±SD of % inactivation and illustrated from a representative experiment performed at least three times.

As shown in table 2, most of the new ligands act by inhibiting the action of SP, and thus can be considered as a new family of SP-antagonists. Analysing these results in depth,some conclusions on the structure-activity relationship can be drawn.Regarding the sulfinyl derivatives, those with a*p*-nitrophenyl substituent at sulfur (*rac*-**10**, *rac*-**11**, *rac*-**12**, *rac*-**13**) present a slightly better inhibitory effect of SP than those with phenyl or *p*-tolyl substituents (table 2, compare entries 24*vs*7, 25*vs*10, 26*vs*16and30,and27*vs*4and29). On the other hand, the substitution of the aromatic ring at the C4 position has a great influence in activity. According to Klebe's model (Figure 4),[21]this ring will be in proximity to Ile204 and His265 and their interaction wasproposed to be of hydrophobic nature. However, the results obtained with our derivatives can be interpreted based ona new hydrogen bond interaction with His265 since compounds *rac*-**5**, **5**(S_S, R_C),*rac*-**12**and *rac*-**16**,with a *p*-nitrophenyl group at thisposition, have some of the highest antagonist effects. On the contrary, halogenated derivatives *rac*-**3**, **3**(R_S, S_C), **3**(S_S, R_C),*rac*-**4**, **4**(R_S, S_C), **4**(S_S, R_C), and *rac*-**11**showed a decreased inhibitory activity due to the lack of this proposed interaction.

In relation to the derivatization of the amino group at C-2 in the pyran ring as an amide, compounds **18**(S_S,R_C) and **18**(R_S,S_C)show a similar antagonist activity compared to their unprotected derivatives **2**(S_S,R_C) and **2**(R_S,S_C). Thioethers *rac*-**19**, **20***R*, **21***R*, which also present an amide at this position, also present comparable activities to their unprotected sulfoxide derivatives [*rac*-**2**, **5**(S_S,R_C) and**1**(S_S,R_C) respectively]. This fact could indicate that the

interaction of the NH_2 group with the Tyr 266 residue is not extremely relevant for the antagonist activity.

When the pyridine ring at C-6 was substituted by other aromatic rings, such as furyl or bis-3,5-(trifluoromethyl)phenyl, (compounds *rac*-**8** and *rac*-**9**,respectively), there was a significant decrease on the antagonist activity. This fact can be explained as the consequence of the suppression of the previously proposed stabilizing interaction between the nitrogen and the His197 residuefor pyridine derivatives. In the case of phenyl derivative *rac*-**9**, together with the significant increase in the steric volume due to the presence of both CF_3 groups in meta positions, the strongly electron-withdrawing effect of these substituents significantly decreases the electronic density of the aromatic ring, thus disfavouring the proposed amino-aromatic interaction between the His197 and the aromatic ring. In the case of the furyl derivative *rac*-**8**, it is also important to consider the low solubility of the furan derivative in the cell media, so that it cannot be used at higher concentrations.

One of the salient features of the results summarized in table 2 is the different antagonist activity showed by the different enantiomer partners. In this sense, all the 5-arylsulfinyl-2-amino-4*H*-pyranswith (S_S,R_C) absolute configuration are more active than their (R_S,S_C)enantiomers. This result indicates that NK1R exercises a chiral discrimination toward the 5-arylsulfinyl-2-amino-4*H*-pyran family, as it was previously described for the reference CP-96345.[12]

Finally, it is worth mentioning that the sulfone derivative *rac*-**17** (IC_{50} = 200±10μM) was the most active antagonist, with a 68.5% of inhibition of production of IP1. This compound presented a notable inhibitory effect at concentrations below $5x10^{-5}$ M (see table 2). It is interesting to note that this increased activity of *rac*-**17** may be explained considering the virtual model of NK1R (Figure 6), where the presence of the sulfonylic oxygen allows the appearance

of an additional stabilizing interaction by a hydrogen bond with the terminal amino group of the amide of Gln165, located at a distance of 3.10 Å. Further, the terminal hydroxyl group of Glu193 is at the right distance to form a hydrogen bond with the other oxygen of the sulfone (2.45 Å), instead of the electrostatic interaction proposed for the other derivatives.

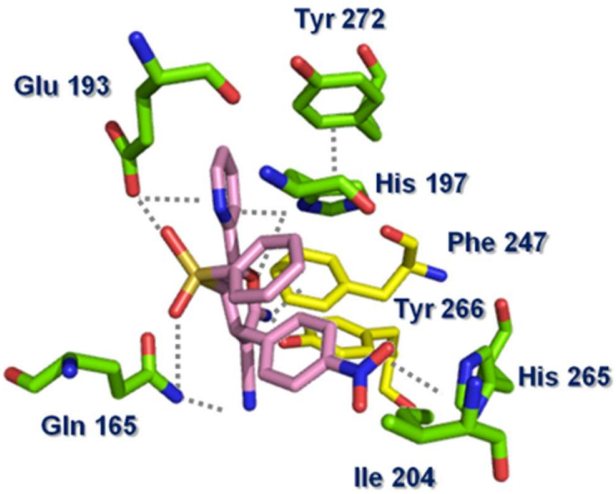


Figure 6. Representation of 5-phenylsulfonyl-2-amino-4*H*-pyran*rac*-**17**in NK1 receptor binding site.

Another interesting result was found when the IPone test was carried out in the absence of SP, in order to determine the agonist activity of the compounds. In this sense, taking into account that structural modifications in close proximity to an oxygen or a nitrogen is likely to affect functional activity of a GPCR ligand,[25] we decided to evaluate the possible interconvertionof our NK1R antagonists into agonists by focusing on modifying the nitrogen at C2. Thus, it was observed that just by acylating the amine in position 2 of the pyran ring, compounds **18**(S_S,R_C),**18**(R_S,S_C), *rac*-**19**,**20***R*, and **21***R* were able to exert a change in the basal activity of the cell, activating NK1R (Figure 7). Notably, up to now, only one non-peptide NK1R agonist has

been reported[36]and was demonstrated to be of therapeutic relevance in angiogenesis-dependent diseases.

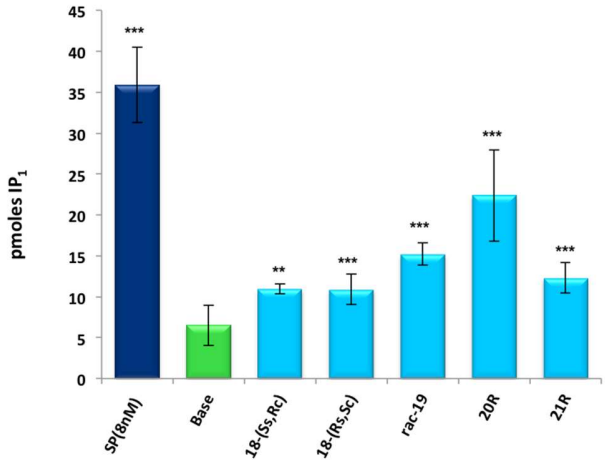


Figure 7. Activation of IP_1 accumulation (pmoles) in NK1 receptor transfected CHO cells by acylated 2-amino-4*H*-pyrans ($5x10^{-5}$M, right) and CP-96345 (10^{-7}M) in the absence of SP. The IP_1 accumulation was measured as described in the experimental section. Data are means ±SD of IP_1 concentration (pmol) and illustrated from a representative experiment performed at least three times. For each nonpeptide compound, a statistical analysis between control and treated cells was done: ** $p<0.01$ and *** $p<0.001$.

As depicted in table 2 and Figure7, all these compounds display both antagonistic and agonistic effects. In the presence of the fully agonist SP, they act as antagonists by blocking access to the receptor, but on their own they act as agonists. These compounds are, thus, partial agonists. Many studies have been published on the benefits of partial agonists,[37]and some current common drugs have been classified as partial agonists, such as buspirone and aripiprazol.[37c]Their clinical use is being increased since they can activate receptors to give a desired submaximal response

when inadequate amounts of the endogenous ligand are present, or they can reduce the overstimulation of receptors when excess amounts of the endogenous ligand are present.[38]Moreover, the use of partial agonists often avoids the development of adverse effects, such as desensitization, adaptation, tolerance and dependence, that are usually associated with overstimulation of the receptors by full agonists.[38]

The analysis of the structure-agonist activity relationships showed that thioethers (*rac*-**19**, **20***R* and **21***R*) were more active than the corresponding sulfoxides [**18**(S_S,R_C) and**18**(R_S,S_C)], with trifluoroacetamides *rac***19** and **20***R* being active even at low concentrations (10^{-5}M) (see supporting information). Compound **20***R*(EC_{50} = 350±22μM) was the best ligand with agonist activity (Figure7), presenting a 62.34 % of activation (see table 2, SI). As in the case of the most potent antagonists, this compound also contains a *p*-nitrophenyl group at position 4 of the pyran ring, which can be responsible for a higher affinity towards the receptor. This confirms that the interaction of the NH_2 group with Tyr 266 and Phe 247 residues must be relevant for the antagonist activity (Figure 4).

2.4. Antitumoral activity evaluation.

Hitherto, all previous studies indicate that NK-1 receptor antagonists selectively inhibit tumour cells proliferation, as well as exerting antiangiogenic and antimetastatic activities.[10a, 39]The overexpression of NK-1 receptors in a wide variety of tumors opens the potential of NK-1 receptor antagonist as a specific treatment against cancer cells, decreasing considerably the side-effects of the treatment.[40]Moreover, their action has been shown to be dose-dependent,with the therapeutic effect being at micromolar range.[6b, 40, 41]Thus, the antitumoral capacity of compounds **1**(S_S,R_C), **1**(R_S,S_C), **6**(S_S,R_C), **6**(R_S,S_C), *rac*-**12**, *rac*-**17**, **18**(S_S,R_C) and *rac*-**19** towards A-549 and

MRC-5 cell lines, was evaluated. In both cases MTT assay and anticancerous activity allowed the determinationof the corresponding IC_{50}.Again, the activity of CP-96345 antagonist was used as a reference. Moreover, lactic acid was used as a reference to study selective toxicity, and the well known anti-cancer drug cisplatin as positive control. Cytotoxicity *vs* concentration line was drawn for each compound (see SI) allowing, thus, the determinationof IC_{50} for healthy and cancerous cells.

Table 3. IC_{50} values of different ligands for A549 y MRC-5 cell lines.

IC_{50}±SD (μM)[a]				
Entry	Compound	A-549	MRC-5	pvalue[d]
1	**CP-96345**	46.83 ± 19.88	57.90 ± 18.03	0.410
2	**Lactic acid**	26.60 ± 3.52[b]	23.93 ± 2.04[b]	0.181
3	**Cisplatin**	11.67 ±7.10	115.71 ±85.66	0.083
4	**1**(S_S,R_C)	172.22 ± 2.68	132.38 ± 30.10	0.184
5	**1**(R_S,S_C)	195.64 ± 4.02	424.68 ± 95.35	0.050
6	**6**(S_S,R_C)	156.42 ± 4.20	429.66 ± 59.54	0.003
7	**6**(R_S,S_C)	507.84 ± 22.08	---	---
8	**18**(S_S,R_C)	107.68 ± 23.04	136.19 ± 32.43	0.232
9	*rac*-**19**	76.47 ± 12.92	130.17 ± 12.71	0.013
10	*rac*-**12**	536.18 ± 54.38	824.57 ± 313.89	0.196
11	*rac*-**17**	148.29 ± 25.73	140.36 ± 41.28	0.811

[a]The$IC_{50}$was calculated as described in the experimental section.[b]Corresponding values for IC_{50}±SD (μM). [c]Data are means ±SD of IC_{50} and illustrated from a representative experiment performed at least three times. [d]For each compound, a statistical analysis between A-549 and MRC-5 trated cells was done.

CP-96345 IC_{50} was around 50 μM for both cell lines. Therefore, no cancerous cell selectivity was observed. Lower concentrations to IC_{50} (Figure 2, SI) exhibited some selectivity.Our

derivatives showed diverse behaviors. Amide derivatives **18**(S_S,R_C) and *rac*-**19**, showed IC_{50} close to 100 μM for the cancerous cell line, highlighting a certain selectivityin the case of derivative *rac*-**19** (Figure 3, SI).Unfortunately, sulfone *rac*-**17** and the *p*-nitrophenylsulfinyl derivative *rac*-**12** (Figure 4, SI) were notselective against A549 cancerous cell line. Moreover, IC_{50} of compound *rac*-**12** was especially high.Better results were found for sulfoxide **1**(R_S,S_C) and **6**(S_S,R_C)and**6**(R_S,S_C) enantiomers (Figures 5 and 6, SI). Although **1**(S_S,R_C) isomer did not present any promising result, its enantiomer **1**(R_S,S_C) showed a good selectivity against lung cancerous cells, even at higher concentrations than its IC_{50}, being able to reach up to 80% of cell death for the cancer line in comparison to less than 30% for healthy cells, at the same concentration. Enantiomers **6**(S_S,R_C) and **6**(R_S,S_C) showed even better results. Namely, 2-amino-4*H*-pyran **6**(S_S,R_C) exhibited IC_{50} values of 156.42±2.10μM for A549 and 429.66±29.77μM for MRC-5 (Table 3). These results indicate a high selectivity against lung cancer cells. At concentrations of 320μM, cell viability of MRC-5 was 70% compared to 7% for A549.It is also important to point out the results obtained for **6**(R_S,S_C) with a IC_{50}(A549) of 507.84±12.75μM. This value is much higher than that obtained for CP-96345, however, no IC_{50} could be obtained for MRC-5 cell line, since at concentrations higher than 1000 μM, the ligand precipitates in cell media. At this high concentration, only 30% of healthy cells inhibition was observed compared to 100% inhibition of cancerous cells. This result showed, therefore, an excellent cancerous selectivity for **6**(R_S,S_C) (Figure 8).

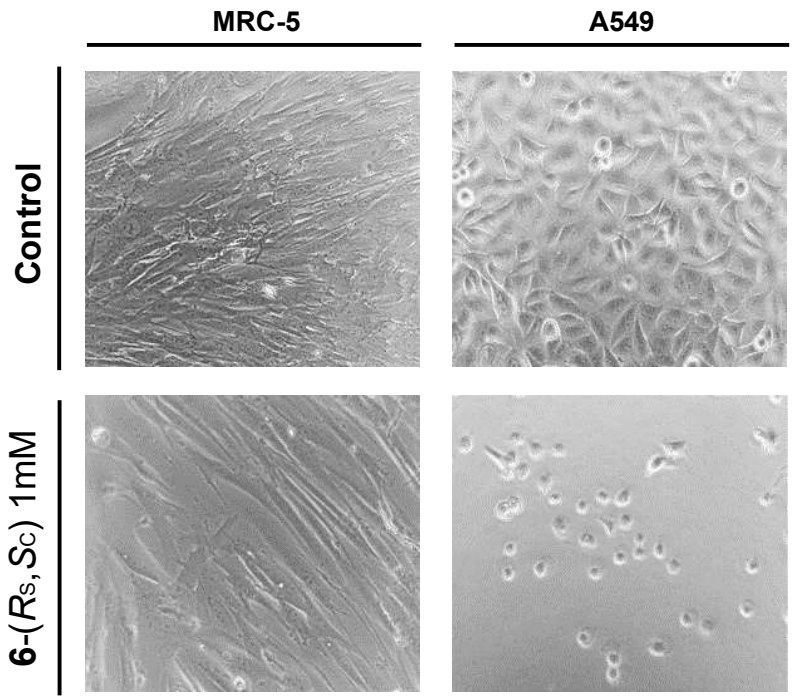


Figure 8. A) Inverted microscope images of human A-549 lung cancer cells and MRC-5 non-malignant lung fibroblasts untreated (control) and incubated with derivative **6**(R_S,S_C) (1mM))

3. Conclusions

In conclusion, a small library of new NK1R ligands with the 5-arylthiosubstituted 2-amino-4*H*-pyran structure has been developed.Sulfonyl derivative *rac*-**17** has proven to inhibit up to 84% of SP activity in a micromolar range and it represents the lead compound for the design of new potent antagonists. Moreover, a new family of non-peptide partial agonists has also been described by the slight modification of the antagonist structure, from the 2-amino to the 2-amide derivative. This is in concordance with an important role of the two new interactions between the NH_2 group and the Phe247 and Tyr 266 residues, proposed in the case of antagonists. The

anticancer activity studies assessed by using human A-549 lung cancer cells and MRC-5 non-malignant lung fibroblasts, revealed a statistically significant high selective cytotoxic effect of some of these 2-amino-4*H*-pyran derivatives toward the lung cancer cells. These promising results could be the adequate starting point for the development of new and more potent NK1 antagonists as anticancerous leads.

4. Experimental section

4.1. Chemistry. General Chemistry Methods.

All reactions were conducted under an atmosphere of dry argon using oven-dried glassware and freshly distilled and dried solvents. TLC was performed on Silica Gel GF254 (Merck) with detection by charring with phosphomolybdic acid/EtOH. For flash chromatography, silica Gel (Merck 230-400 mesh) was used. Chromatographic columns were eluted with positive air pressure and eluents are given as volume to volume ratios (v/v). NMR spectra were recorded with a Bruker Avance DRX500 (^{1}H, 500 MHz), and Bruker AMX500 (^{1}H, 500 MHz) spectrometers. Chemical shifts are reported in ppm, and coupling constants are reported in Hz. Routine spectra were referenced to the residual proton or carbon signals of the solvent. High Resolution mass spectra (HRMS) were recorded in "Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla" with a Kratos MS-80RFA 241-MC apparatus. Optical rotations were determined with a Perkin-Elmer 341 polarimeter. Elemental analyses were measured in a LECO TruSpec® CHNS-932 apparatus. Melting points were measured in STUART SMP3 apparatus in open end capillary tubes.

4.1.1. Synthesis of 5-arylthioderivatives of 2-amino-3-cyano-4,6-diaryl-4H-pyrans.

4.1.1.1. NK-1 antagonists. General Procedure

To a solution of the corresponding β-keto thioderivative (0.38 mmol, 1 eq.) and the appropriate Michael acceptor (0.76 mmol, 2 eq.) in ether or methanol (5-20 mL), a catalytic amount of piperidine is added as base. After stirring overnight, the final product is isolated as a solid by simple filtration, followed by washing with ether. In general, all the products are obtained in good purity, and can be recrystallized from ethanol.

rac-2-Amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,rac-**1**

Prepared following the general procedure from the racemic (*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and benzylidenemalononitrile **28** (117.17 mg, 0.76 mmol) in ether (5mL). The product *rac*-**1** is obtained as a white solid (144.72 mg, 0.35 mmol) in 92% yield. M.p.: 227-228 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.72 (d, *J* = 4.6 Hz, 1H), 8.11 (td, *J* = 1.6 and 7.6 Hz, 1H), 8.07 (d, *J* = 7.8 Hz,1H), 7.60 (t, *J* = 6.0 Hz,1H), 7.45 (d, *J* = 8.2 Hz,2H), 7.25 (s, 2H), 6.93 (m, 3H), 6.87 (d, *J* = 8.1 Hz, 2H), 6.76 (d, *J* = 6.3 Hz, 2H), 4.66 (s,1H), 2.13 (s,3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 160.2, 149.5, 148.7, 148.1, 143.2, 139.5, 139.4, 137.7, 128.6, 128.0, 126.8, 126.1, 125.6, 125.2, 123.6, 122.7, 119.6, 59.2, 32.2, 20.6 ppm. HRMS Calc. for $C_{24}H_{20}N_3O_2S$: $[M+H]^+$ 414.1276, found 414.1253 (-5.6 ppm).Anal. Calc. for $C_{24}H_{19}N_3O_2S$:C, 69.71; H, 4.63; N, 10.16; S, 7.75. Found. C, 68.75; H, 4.63; N, 9.99; S, 7.88.

*(R_S,S_C)-2-Amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,***1***(R_S,S_C)*

Prepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and benzylidenemalononitrile **28** (117.2 mg, 0.76 mmol) in ether (5mL). The product **1**(R_S,S_C) is obtained as a white solid (146.13 mg, 0.35 mmol) in 93% yield. The spectroscopic data are similar to those of the *rac*-**1**. M.p.: 213-215 ºC. HRMS Calc. for $C_{24}H_{20}N_3O_2S$: $[M+H]^+$ 414.1276, found 414.1272 (-1.0 ppm).$[\alpha]^{20}_D$: +198.6 (*c* 0.37, chloroform)

*(S_S,R_C)-2-Amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,***1***(S_S,R_C)*

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and benzylidenemalononitrile **28** (117.2 mg, 0.76 mmol) in ether (5mL). The product **1**(S_S,R_C) is obtained as a white solid (141.41 mg, 0.34 mmol) in 90% yield. The spectroscopic data are similar to those of the **1**(R_S,S_C) enantiomer. M.p.: 214-215 ºC. HRMS Calc. for $C_{24}H_{19}N_3O_2SNa$: $[M+Na]^+$ 436.1096, found 436.1096 (0.1 ppm).$[\alpha]^{20}_D$: -203.2 (*c* 0.37, chloroform)

*rac-2-Amino-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,rac-**2***.

Prepared following the general procedure from the racmic (*p*-tolylsulfinyl)methyl 2-pyridyl ketone *rac*-**22** (98.5 mg, 0.38 mmol) and*p*-methylbenzylidenemalononitrile **29** (127.83 mg, 0.76 mmol) in ether (8 mL). The product *rac*-**2** is obtained as a yellow solid (161 mg, 0.38 mmol) in quantitative yield. M.p.: 218-219 ºC. ^{1}H NMR (500MHz, DMSO-d_6) δ 8,69 (d, *J* = 4.7 Hz, 1H), 8.09 (td, *J* = 1.6 and 7.7 Hz, 1H), 8.04 (d, *J* = 7.9 Hz, 1H), 7.58 (ddd, *J* = 1.3, 4.9 and 7.3 Hz, 1H), 7.41 (d, *J* = 8.2 Hz, 2H), 7.20 (s, 2H), 6.86 (d, *J* = 8.1 Hz, 2H), 6.70 (d, *J* = 7.8 Hz, 2H), 6.61 (d, *J* = 8.0 Hz, 2H), 4.61 (s, 1H), 2.15 (s, 3H), 2.10 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 160.0, 149.1, 148.7, 148.2, 140.2, 139.6, 139.3, 137.6, 135.4, 128.5, 128.4, 126.8, 125.5, 125.2, 123.7, 122.6, 119.6, 72.1, 59.1, 48.7, 31.9, 26.8, 20.6, 20.5 ppm. HRMS Calc. for $C_{25}H_{21}N_3O_2S$: $[M]^+$ 427.1354, found 427.1381 (6.2 ppm).Anal. Calc. for $C_{25}H_{21}N_3O_2S$: C, 70.24; H, 4.95; N, 9.83; S, 7.50. Found. C, 69.78; H, 4.93; N, 9.71; S, 8.02

*(R_S,S_C)-2-Amino-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**2**(R_S,S_C)*.

Prepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and*p*-methylbenzylidenemalononitrile **29** (127.83 mg, 0.76 mmol) in ether (8 mL). The product **1** is obtained as a yellow solid (160 mg, 0.38 mmol) in quantitative

yield.The spectroscopic data are similar to those of the *rac*-**2**. M.p.: 186-187 ºC. HRMS Calc. for $C_{25}H_{22}N_3O_2S$: $[M+H]^+$ 428.1433, found 428.1437 (1.0 ppm).$[\alpha]^{20}_D$: +158.8 (*c* 0.3, chloroform)

*(S$_S$,R$_C$)-2-Amino-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**2**(S$_S$,R$_C$)*.

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and*p*-methylbenzylidenemalononitrile **29** (127.83 mg, 0.76 mmol) in ether (8 mL). The product **2**(S_S,R_C) is obtained as a yellow solid (162 mg, 0.38 mmol) in quantitative yield.The spectroscopic data are similar to those of the **2**(R_S,S_C)enantiomer. M.p.: 182-183 ºC. HRMS Calc. for $C_{25}H_{22}N_3O_2S$: $[M+H]^+$ 428.1433, found 428.1448 (3.6 ppm).$[\alpha]^{20}_D$: -167.3 (*c* 0.3, chloroform)

*rac-2-Amino-3-cyano-4-p-chloroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,rac-**3***

Prepared following the general procedure from the racemic(*p*-tolylsulfinyl)methyl 2-pyridyl ketone *rac*-**22** (98.5 mg, 0.38 mmol) and y *p*-chlorobenzylidenemalononitrile **30** (143.35 mg, 0.76mmol) in ether (10 mL). The product *rac*-**3** is obtained as a white solid (170.22 mg, 0.38 mmol) in quantitative yield. M.p.: 225-227 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.74 (ddd, *J* = 0.9, 1.6 and 4.8 Hz, 1H), 8.13 (td, *J* = 1.7 and 7.7 Hz, 1H), 8.08 (dt, *J* = 1.1 and 7.9 Hz, 1H), 7.62 (ddd, *J* = 1.3, 4.8 and 7.4 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 2H), 7.29 (s, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 6.77 (d, *J* = 8.4 Hz, 2H), 4.69 (s, 1H), 2.22 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.9, 149.3, 148.7, 148.0, 142.0, 139.6, 139.5, 137.6, 131.1, 128.8, 128.6, 127.9, 125.6, 125.2, 123.0, 122.7, 119.3, 72.1, 58.4, 48.7, 31.7, 26.8, 20.6 ppm.HRMS Calc. for $C_{24}H_{18}N_3O_2NaSCl$: $[M+Na]^+$ 470.0706, found 470.0710 (0.9 ppm).Anal. Calc. for $C_{24}H_{18}ClN_3O_2S$: C, 64.35; H, 4.05; N, 9.38; S, 7.16. Found. C, 64.18; H, 4.03; N, 9.38; S, 7.18.

*(R$_S$,S$_C$)-2-Amino-3-cyano-4-p-chloroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**3**(R$_S$,S$_C$)*

IPrepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and y *p*-chlorobenzylidenemalononitrile **30** (143.35 mg, 0.76mmol) in ether (10 mL). The product **3**(R_S,S_C) is obtained as a white solid (167.98 mg, 0.38 mmol) in quantitative yield.The spectroscopic data are similar to those of the *rac*-**3**. M.p.: 186-187 ºC. HRMS Calc. for $C_{24}H_{19}N_3O_2SCl$: $[M+H]^+$ 448.0887, found 448.0880 (-1.5 ppm).$[\alpha]^{20}_D$: +185.3 (*c* 0.32, chloroform).

(*S_S,R_C*)-*2-Amino-3-cyano-4-p-chloroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,***3**(*S_S,R_C*)

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and y *p*-chlorobenzylidenemalononitrile **30** (143.35 mg, 0.76mmol) in ether (10 mL). The product **3**(S_S,R_C) is obtained as a white solid (165 mg, 0.37 mmol) in quantitative yield.The spectroscopic data are similar to those of the **3**(R_S,S_C)enantiomer. M.p.: 186-188 ºC. HRMS Calc. for $C_{24}H_{19}N_3O_2SCl$: $[M+H]^+$ 448.0887, found 448.0892 (1.2 ppm).$[\alpha]^{20}_D$: -194.7 (*c* 0.32, chloroform).

*rac-2-Amino-3-cyano-4-p-fluoroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,rac-***4**

Prepared following the general procedure from the racemic(*p*-tolylsulfinyl)methyl 2-pyridyl ketone *rac*-**22** (98.5 mg, 0.38 mmol) and *p*-fluorobenzylidenemalononitrile **31** (130.84 mg, 0.76mmol) in ether (7 mL). The product *rac*-**4** is obtained as a pink solid (134.45 mg, 0.31 mmol) corresponding to a mixture of both diastereoisomers (R_SS_C, S_SR_C) and (R_SR_C, S_SS_C) in a 86:14 ratio. The residue was recrystallized from ethanol obtaining the major diastereoisomer (R_SS_C, S_SR_C) (98.38 mg, 0.23 mmol) in 60% yield. M.p.: 213-214 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.73 (dt, *J* = 1,2 and 4.8 Hz, 1H), 8.12 (td, *J* = 1.7 and 7.7 Hz, 1H), 8.08 (dt, *J* = 1.0 and 7.9 Hz, 1H), 7.62 (ddd, *J* = 1.5, 4.8 and 7.3 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 2H), 7.26 (s, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 6.82-6.78 (m, 2H), 6.76-6.72 (m, 2H), 4.71 (s, 1H), 2.19 (s, 3H)

ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 161.7, 160.0, 159.8, 149.2, 148.6, 148.0, 139.6, 139.4, 137.6, 128.9, 128.8, 128.5, 125.5, 125.2, 123.3, 122.7, 119.4, 114.7, 114.5, 58.8, 31.5, 20.5 ppm.HRMS Calc. for $C_{24}H_{19}N_3O_2FS$: $[M+H]^+$ 432.1182, found 432.1182 (0.0 ppm).

(*R_S,S_C*)-*2-Amino-3-cyano-4-p-fluoroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**4***(R_S,S_C)

Prepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and *p*-fluorobenzylidenemalononitrile **31** (130.84 mg, 0.76mmol) in ether (7 mL). The product **4**(R_S,S_C) is obtained as a pink solid (139.37 mg, 0.32 mmol) in 85% yield.The spectroscopic data are similar to those of the *rac*-**4**. M.p.: 207-208 ºC.HRMS Calc. for $C_{24}H_{19}N_3O_2FS$: $[M+H]^+$ 432.1182, found 432.1173 (-2.1 ppm).$[\alpha]^{20}_D$: +202.2 (*c* 0.34, chloroform).

(*S_S,R_C*)-*2-Amino-3-cyano-4-p-fluoroyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**4**(S_S,R_C)*

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and *p*-fluorobenzylidenemalononitrile **31** (130.84 mg, 0.76mmol) in ether (7 mL). The product **4**(S_S,R_C) is obtained as a pink solid (136.10 mg, 0.32 mmol) in 85% yield.The spectroscopic data are similar to those of the **4**(R_S,S_C)enantiomer. M.p.: 206-207 ºC.HRMS Calc. for $C_{24}H_{19}N_3O_2FS$: $[M+H]^+$ 432.1182, found 432.1177 (-1.2 ppm).$[\alpha]^{20}_D$: -194.4 (*c* 0.34, chloroform).

*rac-2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-p-tolylsulfinyl -4H-pyran,rac-**5***

Prepared following the general procedure from the racemic(*p*-tolylsulfinyl)methyl 2-pyridyl ketone *rac*-**22** (98.5 mg, 0.38 mmol) and y *p*-nitrobenzylidenemalononitrile **32** (151.37 mg, 0.76mmol) in ether (20 mL). The product *rac*-**5** is obtained as a brown solid (137.64 mg, 0.30 mmol) in 79% yield. M.p.: 238-239 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.75 (d, *J* = 4.5 Hz, 1H), 8.15 (td, *J* = 1.3 and 7.8 Hz, 1H), 8.10 (d, *J* = 7.8 Hz, 1H), 7.79 (d, *J* = 8.6 Hz, 2H), 7.65 (t,

J = 6.0Hz, 1H), 7.48 (d, *J* = 8.1 Hz, 2H), 7.41 (s, 2H), 7.04 (d, *J* = 8.6 Hz, 2H), 6.90 (d, *J* = 8.1 Hz, 2H), 4.84 (s, 1H), 2.10 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 160.0, 150.5, 149.6, 148.8, 147.9, 145.6, 139.8, 139.4, 137.7, 128.7, 128.5, 125.8, 125.2, 123.2, 122.9, 122.4, 119.1, 57.5, 32.2, 20.4 ppm.HRMS Calc. for $C_{24}H_{18}N_4O_4NaS$: $[M+Na]^+$ 481.0946, found 481.0961 (3.0 ppm).Anal. Calc. for $C_{24}H_{18}N_4O_4S$: C, 62.87; H, 3.96; N, 12.22; S, 6.99. Found. C, 62.36; H, 3.97; N, 11.84; S, 7.40.

(*R_S,S_C*)-*2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**5**(R_S,S_C)*

Prepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and y *p*-nitrobenzylidenemalononitrile **32** (151.37 mg, 0.76mmol) in ether (20 mL). The product **5**(R_S,S_C) is obtained as a brown solid (141.12 mg, 0.31 mmol) in 81% yield. The spectroscopic data are similar to those of the *rac*-**5**. M.p.: 216-218 ºC.HRMS Calc. for $C_{24}H_{19}N_4O_4S$: $[M+H]^+$ 459.1127, found 459.1103 (-5.2 ppm). $[\alpha]^{20}_D$: +230.0 (*c* 0.3, chloroform).

*(S_S,R_C)-2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,**5**(S_S,R_C)*

IPrepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and y *p*-nitrobenzylidenemalononitrile **32** (151.37 mg, 0.76mmol) in ether (20 mL). The product **5**(S_S,R_C) is obtained as a brown solid (144.61 mg, 0.32 mmol) in 83% yield. The spectroscopic data are similar to those of the**5**(R_S,S_C)enantiomer. M.p.: 216-218 ºC.HRMS Calc. for $C_{24}H_{18}N_4O_4SNa$: $[M+Na]^+$ 481.0946, found 481.0947 (0.1 ppm). Anal. Calc. for $C_{24}H_{18}N_4O_4S$: C, 62.87; H, 3.96; N, 12.22; S, 6.99. Found. C, 63.01; H, 4.01; N, 12.01; S, 6.93. $[\alpha]^{20}_D$: -244.7 (*c* 0.3, chloroform).

*rac-2-Amino-3-cyano-4-p-methoxyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran,rac-**6***

Prepared following the general procedure from the racemic (*p*-tolylsulfinyl)methyl 2-pyridyl ketone *rac*-**22** (98.5 mg, 0.38 mmol) and y *p*-methoxybenzylidenemalononitrile **33** (140 mg, 0.76mmol) in ether (7mL). The product *rac*-**6** is obtained as a white solid (137.49 mg, 0.31 mmol) in 82% yield. M.p.: 213-214 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.69 (d, *J* = 4.3 Hz, 1H), 8.09 (td, *J* = 1.5 and 7.7 Hz, 1H), 8.05 (d, *J* = 7.9 Hz,1H), 7.58 (ddd, *J* = 1.3, 4.9 and 7.3 Hz,1H), 7.42 (d, *J* = 8.2 Hz,2H), 7.20 (s, 2H), 6.90 (d, *J* = 8.0 Hz, 2H), 6.65 (d, *J* = 8.6 Hz, 2H), 6.46 (d, *J* = 8.6 Hz, 2H), 4.61 (s,1H), 3.60 (s,3H), 2.16 (s,3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.9, 157.9, 148.9, 148.6, 148.2, 139.8, 139.1, 137.6, 135.3, 128.5, 128.1, 125.4, 125.2, 123.8, 122.6, 119.6, 113.4, 59.2, 55.0, 31.6, 20.5 ppm.HRMS Calc. for $C_{25}H_{21}N_3O_3NaS$: $[M+Na]^+$ 466.1201, found 466.1207 (1.2 ppm).Anal. Calc. for $C_{25}H_{21}N_3O_3S$: C, 67.70; H, 4.77; N, 9.47; S, 7.23. Found. C, 62.94; H, 4.68; N, 8.52; S, 6.65.

(R_S,S_C)-2-Amino-3-cyano-4-p-methoxyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran, ***6****(R_S,S_C)*

Prepared following the general procedure from the (*S*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***S* (98.5 mg, 0.38 mmol) and y *p*-methoxybenzylidenemalononitrile **33** (140 mg, 0.76mmol) in ether (7mL). The product **6**(R_S,S_C)is obtained as a white solid (131.79 mg, 0.30 mmol) in 80% yield.The spectroscopic data are similar to those of the *rac*-**6**. M.p.: 188-189 ºC. HRMS Calc. for $C_{25}H_{22}N_3O_3S$: $[M+H]^+$ 444.1382, found 444.1375 (-1.6 ppm).$[\alpha]^{20}_D$: +185.5 (*c* 0.32, chloroform).

(S_S,R_C)-2-Amino-3-cyano-4-p-methoxyphenyl-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran, ***6****(S_S,R_C)*

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.5 mg, 0.38 mmol) and y *p*-methoxybenzylidenemalononitrile **33** (140 mg, 0.76mmol) in ether (7mL). The product **6**(S_S,R_C) is obtained as a white solid (143.3 mg, 0.32 mmol) in 85% yield. The spectroscopic data are similar to those of the **6**(R_S,S_C) enantiomer. M.p.: 187-188 ºC.

HRMS Calc. for $C_{25}H_{21}N_3O_3NaS$: $[M+Na]^+$ 466.1201, found 466.1230 (6.2 ppm).Anal. Calc. for $C_{25}H_{21}N_3O_3S$: C, 67.70; H, 4.77; N, 9.47; S, 7.23. Found. C, 67.63; H, 4.63; N, 9.38; S, 7.53.$[\alpha]^{20}_D$: -175.6 (*c* 0.32, chloroform).

(S_S,R_C)-2-Amino-3-cyano-4-[3,5-bis(trifluoromethyl)phenyl]-6-(2-pyridyl)-5-p-tolylsulfinyl-4H-pyran, ***7****(S_S,R_C)*

Prepared following the general procedure from the (*R*)-(*p*-tolylsulfinyl)methyl 2-pyridyl ketone **22***R* (98.50 mg, 0.38 mmol) and 3,5-bis(trifluoromethyl)benzylidenemalononitrile **34** (220.52 mg, 0.76mmol) in ether (6 mL). The product **7**(S_S,R_C) is obtained as a white solid (146.16 mg, 0.27 mmol) in 70% yield. M.p.: 229-231 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.73 (ddd, *J* = 0.9, 1.5 and 4.8 Hz, 1H), 8.13 (td, *J* = 1.7 and 7.8 Hz, 1H), 8.08 (dt, *J* = 1.0 and 7.9 Hz, 1H), 7.67 (s, 1H), 7.63 (ddd, *J* = 1.3, 4.8and 7.4 Hz, 1H), 7.46 (d, *J* = 8.3 Hz, 2H), 7.41-4.40 (m,4H), 6.83 (d, *J* = 8.0 Hz, 2H), 5.03 (s,1H), 2.09 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.9, 151.9, 148.9, 140.8, 139.8, 137.6, 135.3, 131.2, 103.6, 129.2, 127.1, 124.7 (c, *J*= 135.4 Hz, C-F), 122.6, 120.1, 119.6, 113.4, 59.2, 55.1, 31.7, 20.4 ppm.HRMS Calc. for $C_{26}H_{17}N_3O_2SF_6$: $[M]^+$ 549.0930, found 549.0946 (-2.9 ppm).$[\alpha]^{20}_D$: -184.4 (*c* 0.4, chloroform).

*rac-2-Amino-3-cyano-4-p-chloroyphenyl-6-(2-furyl)-5-p-tolylsulfinyl-4H-pyran,rac-***8**

Prepared following the general procedure from the racemic(*p*-tolylsulfinyl)methyl 2-furyl ketone *rac*-**23** (94.35 mg, 0.38 mmol) and *p*-chlorobenzylidenemalononitrile **30** (143.35 mg, 0.76mmol) in ether (12 mL). The product *rac*-**8** is obtained as a white solid (137.80 mg, 0.32 mmol) in 83% yield. M.p.: 243-244 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.04 (dd, *J* = 0.9 and 1.8 Hz, 1H), 7.22 (dd, *J* = 0.7 and 3.6 Hz, 1H), 7.21 (s, 2H), 7.18 (d, *J* = 9.1 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 4H), 6.82 (dd, *J* = 1.8 and 3.6 Hz, 1H), 6.60 (d, *J* = 9.1, 2H)2.19 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.9, 147.4, 144.4, 143.6, 142.1, 140.8, 137.9, 131.4, 129.5, 128.9, 128.3, 124.3,

119.5, 119.0, 115.5, 112.8, 59.0, 32.4, 20.9 ppm.HRMS Calc. for $C_{23}H_{18}N_2O_3SCl$: $[M+H]^+$ 437.0727, found 437.0697 (-6.8 ppm).

*rac-2-Amino-3-cyano-4-p-tolyl-6-[3,5-bis(trifluoromethyl)phenyl]-5-p-tolylsulfinyl-4H-pyran,rac-**9***

Prepared following the general procedure from the racemic(*p*-tolylsulfinyl)methyl3,5-bis(trifluoromethyl) phenyl ketone *rac*-**24** (149.85 mg, 0.38 mmol) and *p*-methylbenzylidenemalononitrile **29** (127.83 mg, 0.76mmol) in ether (6 mL). The product *rac*-**9** is obtained as a white solid (171 mg, 0.30 mmol) in 80% yield. M.p.: 230-231 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.47 (s, 2H), 8.40 (s, 1H), 7.44 (d, *J* = 8.3 Hz, 2H), 7.39 (d, *J* = 8.2 Hz, 2H), 7.19 (s, 2H), 7.15 (d, *J* = 7.9 Hz, 2H), 7.08 (d, *J* = 7.9 Hz, 2H), 3.74 (s,1H), 2.42 (s, 3H), 2.31 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.4, 151.9, 141.4, 140.8, 138.0, 136.2, 133.0, 130.8, 130.6, 130.0, 129.2, 127.1, 124.7, 123.1, 121.9, 119.1, 58.8, 37.2, 26.8, 20.9, 20.6 ppm.HRMS Calc. for $C_{28}H_2N_2O_2SF_6$: $[M+H]^+$ 563.1228, found 563.1252 (4.3 ppm).

*rac-2-Amino-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-p-nitrophenylsulfinyl-4H-pyran,rac-**10***

It is prepared following the general procedure from the racemic(*p*-nitrophenylsulfinyl)methyl 2-pyridyl ketone*rac*-**26** (110.31 mg, 0.38 mmol) and *p*-methylbenzylidenemalononitrile **29** (127,83 mg, 0.76mmol) in ether (20 mL). The product *rac*-**10** is obtained as a brown solid (123.80 mg, 0.27 mmol) in 70% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 214-215 ºC. ^{1}H NMR (500 MHz, DMS-d_6O) δ: 8.69 (ddd, *J* = 1.0, 1.6 and 4.8 Hz, 1H), 8.13 (td, *J* = 1.6 and 8.7 Hz, 1H), 8.09 (d, *J* = 7.9 Hz, 1H), 7.89 (d, *J* = 9.0 Hz, 2H),7.80 (d, *J* = 9.0 Hz, 2H), 7.62 (ddd, *J* = 1.3, 4.8 and 7.4 Hz, 1H), 7.26 (s,2H), 6.66 (d, *J* = 8.0 Hz, 2H) 6.63 (d, *J* = 8.2 Hz, 2H), 4.62 (s,1H), 2.00 (s, 3H)ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 206.4, 159.5, 151.3, 149.2, 148.6, 147.8, 147.7, 139.7, 137.8, 136.1, 128.5, 127.4, 127.0, 122.8, 122.7,

122.3, 78.9, 58.6, 32.3, 30.7, 20.2ppm.HRMS Calc. for $C_{24}H_{19}N_4O_4S$: $[M+H]^+$ 459.1127, found 459.1125 (-0.4 ppm).

*rac-2-Amino-3-cyano-4-p-chlorophenyl-6-(2-pyridyl)-5-p-nitrophenylsulfinyl-4H-pyran, rac-**11***

Prepared following the general procedure from the racemic(*p*-nitrophenylsulfinyl)methyl 2-pyridyl ketone *rac*-**26** (110.31 mg, 0.38 mmol) and *p*-chlorobenzylidenemalononitrile **30** (143,45 mg, 0.76mmol) in ether (20 mL). The product *rac*-**11** is obtained as a brown solid (91 mg, 0.19 mmol) in 50% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 211-213 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.73 (ddd, *J* = 1.0, 1.6 and 4.8 Hz, 1H), 8.16 (td, *J* = 1.7 and 7.7 Hz, 1H), 8.12 (dt, *J* = 1.2 and 7.7 Hz, 1H), 7.99-7.96 (m, 2H), 7.88-7.85 (m, 2H), 7.66 (ddd, *J* = 1.4, 4.8 and 7.4 Hz, 1H), 7.35 (s, 2H), 6.96-6.93 (m, 2H), 6.85-6.82 (m, 2H), 4.73 (s,1H)ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.6, 151.3, 149.4, 148.7, 147.8, 147.7, 141.4, 137.9, 131.6, 129.4, 128.0, 127.2, 125.9, 123.0, 122.8, 121.6, 119.2, 58.0, 32.0 ppm.HRMS Calc. for $C_{23}H_{16}N_4O_4SCl$: $[M+H]^+$ 479.0581, found 479.0589 (1.7 ppm).

*rac-2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-p-nitrophenylsulfinyl-4H-pyran,rac-**12***

Prepared following the general procedure from the racemic(*p*-nitrophenylsulfinyl)methyl 2-pyridyl ketone *rac*-**26** (110.31 mg, 0.38 mmol) and*p*-nitrobenzylidenemalononitrile **32** (151,37 mg, 0.76mmol) in ether (20 mL). The product *rac*-**12** is obtained as a brown solid (108 mg, 0.22 mmol) in 58% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 205-207 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.75 (ddd, *J* = 1.0, 1.5 and 4.8 Hz, 1H), 8.18 (td, *J* = 1.7 and 7.8 Hz, 1H), 8.15 (dt, *J* = 1.2 and 7.8 Hz, 1H), 7.91 (d, *J* = 9.1 Hz, 2H),7.87 (d, *J* = 9.1 Hz, 2H),7.75 (d, *J* = 8.7 Hz, 2H), 7.68 (ddd, *J* = 1.5, 4.8 and 7.2 Hz, 1H), 7.47 (s,2H), 7.11(d, *J* = 8.7 Hz, 2H), 4.88 (s,1H)ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.6, 151.1, 149.8 (2C), 148.6, 147.9, 147.6, 145.9, 137.9, 128.9, 128.1, 127.2, 126.0, 123.2, 122.9, 122.8,

121.0, 118.9, 57.2, 32.4, 30.6ppm.HRMS Calc. for $C_{23}H_{16}N_5O_6S$: $[M+H]^+$ 490.0821, found 490.0832 (2.2 ppm).

rac-2-Amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-p-nitrophenylsulfinyl-4H-pyran,rac-**13**

Prepared following the general procedure from the racemic(*p*-nitrophenylsulfinyl)methyl 2-pyridyl ketone *rac*-**26** (110.31 mg, 0.38 mmol) andbenzylidenemalononitrile **28** (117,17 mg, 0.76mmol) in ether (20 mL). The product *rac*-**13** is obtained as a brown solid (93 mg, 0.21 mmol) in 55% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 202-203 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.74 (ddd, *J* = 1.0, 1.6 and 4.8 Hz, 1H), 8.16 (td, *J* = 1.6 and 8.0 Hz, 1H), 8.13 (dt, *J* = 1.3 and 7.4 Hz, 1H), 7.92 (d, *J* = 9.1 Hz, 2H),7.88 (d, *J* = 9.1 Hz, 2H), 7.66 (ddd, *J* = 1.6,4.9 and 7.1 Hz, 1H), 7.30 (s,2H), 6.92-6.89 (m, 3H), 6.81-6.79 (m, 2H), 4.71 (s,1H)ppm. ^{13}C NMR (125.7 MHz, DMS-d_6O) δ: 159.7, 151.2, 149.6, 148.7, 147.9, 147.7, 142.7, 137.8, 128.0, 127.2, 127.0, 126.5, 125.8, 122.8, 122.7, 122.2, 119.3, 58.8, 32.5ppm.HRMS Calc. for $C_{23}H_{16}N_4O_4SNa$: $[M+Na]^+$ 467.0790, found 467.00794 (0.9 ppm).

*rac-2-Amino-3-cyano-4-(p-metoxyphenyl)-6-(2-pyridyl)-5-phenylsulfinyl-4H-pyran,rac-**14***

Prepared following the general procedure from the racemic(phenylsulfinyl)methyl 2-pyridyl ketone,*rac*-**25**, (93.20 mg, 0.38 mmol) and*p*-metoxybenzylidenemalononitrile **33** (140 mg, 0.76mmol) in ether (7 mL). The product *rac*-**14** is obtained as a white solid (100 mg, 0.23 mmol) in 60% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 205-206 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.72 (dt, *J* = 1.2 and 4.5 Hz, 1H), 8.12 (td, *J* = 1.6 and 7.6 Hz, 1H), 8.09 (dt, *J* = 1.2 and = 7.8 Hz, 1H), 7. 61-7.59 (m, 3H), 7.20 (s, 2H), 7.17-7.12 (m, 3H), 6.71 (d, *J* = 8.6 Hz, 2H), 6.47 (d, *J* = 8.7 Hz, 2H), 4.68 (s, 1H), 3.61 (s, 3H)ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 159.9, 157.7, 149.1, 148.6, 148.1, 143.0, 137.6, 135.1, 129.4, 128.0,

127.9, 125.5, 125.2, 123.4, 122.6, 119.5, 113.5, 59.3, 55.0, 31.5ppm.HRMS Calc. for $C_{24}H_{19}N_3O_3NaS$: $[M+Na]^+$ 452.1045, found 452.1052 (1.6 ppm).

*rac-2-Amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-phenylsulfinyl-4H-pyran,rac-**15***

Prepared following the general procedure from the racemic(phenylsulfinyl)methyl 2-pyridyl ketone *rac*-**25** (93.20 mg, 0.38 mmol) andbenzylidenemalononitrile **28** (117.17 mg, 0.76mmol) in ether (5 mL). The product *rac*-**15** is obtained as a white solid (91 mg, 0.23 mmol) in 60% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 205-207 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.75 (ddd, *J* = 1.0, 1.5 and 4.8 Hz, 1H), 8.15-8.09 (m, 2H), 7.65-7.61 (m, 3H), 7.25 (s, 2H), 7.16-7.09 (m, 3H), 6.93 (dd, *J* = 1.8 and 5.0 Hz, 3H), 6.82-6.80 (m, 2H), 4.73 (s, 1H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 160.1, 149.5, 148.6, 148.0, 143.0, 142.7, 137.6, 129.5, 128.0, 127.9, 126.8, 126.4, 125.5, 125.2, 123.1, 122.6, 119.4, 59.2, 32.1, 30.6 ppm.HRMS Calc. for $C_{23}H_{18}N_3O_2S$: $[M+H]^+$ 400.1120, found 400.1103 (-4.0 ppm).

*rac-2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-phenylsulfinyl-4H-pyran,rac-**16***

Prepared following the general procedure from the racemic(phenylsulfinyl)methyl 2-pyridyl ketone,*rac*-**25**, (93.20 mg, 0.38 mmol) and*p*-nitrobenzylidenemalononitrile **32** (151,37 mg, 0.76mmol) in ether (11 mL). The product *rac*-**16** is obtained as a white solid (132.4 mg, 0.3 mmol) in 78% yield,after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 200-201 ºC. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.78-8.75 (m,1H), 8.16-8.11 (m, 2H), 7.78 (d, *J* = 8.8 Hz, 2H), 7.66-7.63 (m, 3H), 7.42 (m, 2H), 7.13-7.11 (m, 3H), 7.08 (d, *J* = 8.8 Hz, 2H) 4.89 (s, 1H)ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 160.1, 150.4, 149.9, 148.8, 147.8, 145.9, 142.7, 137.8, 129.7, 128.5, 128.2, 125.9, 125.3, 123.3, 122.8, 122.0, 119.1, 57.6, 32.2 ppm.HRMS Calc. for $C_{23}H_{17}N_4O_4S$: $[M+H]^+$ 445.0971, found 445.0987 (3.7 ppm).

rac-2-Amino-3-cyano-4-p-nitrophenyl-6-(2-pyridyl)-5-phenylsulfonyl-4H-pyran,rac-**17**

Prepared following the general procedure from the (phenylsulfonyl)methyl 2-pyridyl ketone **27** (70.5 mg, 0.27 mmol) and *p*-nitrobenzylidenemalononitrile **32** (53.8 mg, 0.27 mmol) in ethanol (0.54 mL). The product *rac*-**17**is obtained as a brown solid (55.3, 0.12 mmol) in 43% yield, after purification by flash chromatography (toluene-isopropanol, 15:1). M.p.: 193-194 ºC. ^{1}H NMR (500MHz, DMSO-d_6) δ 8.67 (ddd, *J* = 1.0, 1.6 y 4.8 Hz, 1H), 8.13 (d, *J* = 8.8, 2H), 8.00 (td, *J* = 1.7 and 7.7 Hz, 1H), 7.80 (dt, *J* = 1.0 and 7.8 Hz, 1H), 7.59-7.56 (m, 4H), 7.48 (d, *J* = 8.8, 2H), 7.41-7.38 (m, 2H), 7.35 (s, 2H), 4.73 (s, 1H) ppm. ^{13}C NMR (125.7 MHz, DMSO-d_6) δ: 158.8, 155.5, 150.0, 149.7, 149.0, 146.6, 140.0, 136.7, 133.5, 128.9, 128.6, 127.6, 125.4, 125.3, 123.9, 118.6, 118.4, 57.0 ppm. HRMS Calc. for $C_{23}H_{17}N_4O_5S$: $[M+H]^+$ 461.0920, found 461.0946 (5.7 ppm).

4.1.1.2. NK-1 agonists. General Procedure

4.1.1.2.1. Amide derivatives.

A solution of acetic anhydride (0.17 mmol, 1.5 eq.) and a catalytic amount of DMAP was added to a solution of corresponding 2-amino-4*H*-pyran (0.12 mmol, 1 eq.) in dry pyridine (1.3 mL) at 0 ºC. After stirring for 2 h, the reaction mixture was quenched with 5% HCl aqueous solution (5 mL). The aqueous phase was then extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic phases were washed with saturated aqueous $NaHCO_3$ solution and saturated aqueous NaCl solution, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the crude was purified by flash chromatography (toluene-isopropanol, 20:1)

(R_S,S_C)-2-Acetamide-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-ptolylsulfinyl-4H-pyran, ***18****(R_S,S_C)*

Prepared following the general procedure from the (R_S,S_C)- 2-amino-3-cyano-4-*p*-tolyl-6-(2-pyridyl)-5-*p*-tolylsulfinyl-4*H*-pyran, **2**(R_S,S_C) (50 mg, 0.12 mmol) in pyridine (1.3 mL). The product **18**(R_S,S_C)is obtained as a white solid (40 mg 0.08 mmol) in 70% yield, after purification

by flashchromatography. M.p.: 90-92 ºC. ^{1}H NMR (500MHz, $CDCl_3$) δ 8.63 (ddd, *J* = 0.9, 1.6 and 4.8 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 1H), 8.05 (s, 1H), 7.88 (dt, *J* = 1.7 and 7.8 Hz, 1H), 7.56 (d, *J* = 8.3 Hz, 2H), 7.40 (ddd, *J* = 1.1, 4.8 and 7.6 Hz, 1H), 6.82 (d, *J* = 8.0 Hz, 2H), 6.75 (d, *J* = 8.1 Hz, 2H), 6.69 (d, *J* = 8.0 Hz, 2H), 5.06 (s, 1H), 2.19 (s, 3H), 2.18 (s, 3H), 2.15 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ: 168.6, 152.0, 150.1, 148.7, 148.4, 139.9, 139.7, 137.9, 137.3, 137.0, 129.1, 128.8, 127.8, 125.7, 125.2, 123.5, 123.4, 116.2, 33.0, 29.8, 24.1, 21.2, 21.0 ppm. HRMS Calc. for $C_{27}H_{23}N_3O_3S$: $[M+H]^+$ 469.1470, found 469.1460 (2.1 ppm).$[\alpha]^{20}_D$: +168.8 (*c* 0.3, chloroform).

(S_S,R_C)-2-Acetamide-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-ptolylsulfinyl-4H-pyran, ***18****(S_S,R_C)*

Prepared following the general procedure from the (S_S,R_C)- 2-amino-3-cyano-4-*p*-tolyl-6-(2-pyridyl)-5-*p*-tolylsulfinyl-4*H*-pyran, **2**(S_S,R_C) (50 mg, 0.12 mmol) in pyridine (1.3 mL). The product **18**(S_S,R_C)is obtained as a white solid (42.26 mg 0.09 mmol) in 75% yield, after purification by flashchromatography. M.p.: 88-92 ºC. The spectroscopic data are similar to those of the **18**(R_S,S_C) enantiomer. HRMS Calc. for $C_{27}H_{23}N_3O_3S$: $[M+H]^+$ 469.1446, found 469.1460 (-1.4 ppm).$[\alpha]^{20}_D$: -166.3 (*c* 0.3, chloroform).

4.1.1.2.2. Trifluoroacetyl sulfinyl derivatives.

A solution of trifluoroacetic anhydride (1.20 mmol, 5 eq.) in acetone (0.6) was added to a solution of corresponding 2-amino-4*H*-pyran (0.24 mmol, 1 eq.) and NaI (0.72 mmol, 3 eq.) in acetone (1.2 mL) at -40 ºC. After stirring for 30 min., the reaction mixture was quenched with 20% sodium sulfite aqueous solution (5 mL). The aqueous phase was then extracted with EtOAc (3 x 20 mL) and the combined organic phases were washed with saturated aqueous $NaHCO_3$ solution and saturated aqueous NaCl solution, and dried over anhydrous Na_2SO_4. The solvent

was evaporated under reduced pressure and the crude was purified by flash chromatography (toluene-isopropanol, 15:1)

*rac-2-Trifluoroacetamide-3-cyano-4-p-tolyl-6-(2-pyridyl)-5-ptolylsulfenyl-4H-pyran,rac-**19***

Prepared following the general procedure from the *rac*-2-amino-3-cyano-4-*p*-tolyl-6-(2-pyridyl)-5-*p*-tolylsulfinyl-4*H*-pyran*, rac*-**2** (103 mg, 0.24 mmol) in acetone (1.2 mL). The product *rac*-**19**is obtained as a yellow solid (76.13 mg 0.15 mmol) in 60% yield, after purification by flashchromatography. M.p.: 114-115 ºC. ^{1}H NMR (500MHz, $CDCl_3$) δ 8.70 (dt, *J* = 1.3 and 4.6 Hz, 1H), 7.84-7.80 (m, 2H), 7.36 (ddd, *J*= 1.9, 4.2 and 6.3 Hz, 1H), 7.16-7.11 (m, 4H), 7.08 (d, *J*= 7.9 Hz, 2H), 6.99 (d, *J*= 8.0 Hz, 2H), 4.14 (s, 1H), 2.37 (s, 3H), 2.32 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ: 155.1 (c, *J*= 38.8 Hz, C-F), 149.8, 149.1, 148.8, 145.8, 138.8, 138.3, 137.1, 136.9, 132.4, 130.4, 129.8, 127.9, 127.3, 125.0, 124.6, 116.4, 115.7, 115.3, 85.5, 42.9, 21.4, 21.3 ppm. HRMS Calc. for $C_{27}H_{20}N_3O_2SF_3$: $[M]^+$ 507.1230, found 570.1232 (-0.1 ppm).

*(R)-2-Trifluoroacetamide-3-cyano-4-p-nitrohenyl-6-(2-pyridyl)-5-p-tolylsulfenyl-4H-pyran,**20**R*

Prepared following the general procedure from the (S_S,R_C)- 2-amino-3-cyano-4-*p*-nitrophenyl-6-(2-pyridyl)-5-*p*-tolylsulfinyl-4*H*-pyran*,* **5**(S_S,R_C) (110 mg, 0.24 mmol) in acetone (1.2 mL). The product **20***R*is obtained as a brown solid (91.55 mg 0.17 mmol) in 70% yield, after purification by flashchromatography. M.p.: 96-98 ºC. ^{1}H NMR (500MHz, $CDCl_3$) δ 8.76 (dt, *J* = 1.2 and 4.8, 1H), 8.15 (d, *J* = 8.7, 2H), 7.89-7.83 (m, 2H), 7.42-7.39(m, 1H), 7.25 (d, *J* = 8.8, 2H), 7.10 (s, 4H), 4.36 (s, 1H), 2.37 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ: 154.7 (c, *J*= 40.2 Hz, C-F), 149.6, 149.4, 148.8, 147.9, 146.9, 145.4, 139.7, 137.3, 133.3, 130.5, 129.0, 126.5, 125.5, 124.7, 124.5, 124.3, 115.1, 114.7, 83.5, 43.2, 21.4 ppm. HRMS Calc. for $C_{26}H_{18}N_4O4_2SF_3$: $[M+H]^+$ 539.1001, found 539.1022 (3.9 ppm).$[\alpha]^{20}_D$: -49.2 (*c*1.0, chloroform).

*(R)-2-Trifluoroacetamide-3-cyano-4-phenyl-6-(2-pyridyl)-5-ptolylsulfenyl-4-H-pyran,**21**R*

Prepared following the general procedure from the (S_S,R_C)- 2-amino-3-cyano-4-phenyl-6-(2-pyridyl)-5-*p*-tolylsulfinyl-4*H*-pyran, **1**(S_S,R_C) (100 mg, 0.24 mmol) in acetone (1.2 mL). The product **21***R*is obtained as a green solid (89.24 mg 0.18 mmol) in 75% yield, after purification by flashchromatography. M.p.: 100-103 ºC. ^{1}H NMR (500MHz, $CDCl_3$) δ 8.72 (dt, *J* = 1.3 and 4.8, 1H), 7.82 (d, *J* = 3.5, 2H), 7.30-7.27 (m, 3H), 7.13-7.09 (m, 6H), 4.18 (s, 1H), 2.37 (s, 3H) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ: 155.1 (c, *J*= 40.0 Hz, C-F), 149.8, 149.2, 148.7, 145.5, 140.0, 139.0, 137.1, 135.4, 132.8, 130.3, 129.1, 128.4, 128.0, 124.9, 124.5, 116.4, 115.7, 115.2, 85.4, 43.4, 21.3 ppm. HRMS Calc. for $C_{26}H_{18}N_3O_2SF_3$: $[M]^+$ 493.1072, found 493.1072 (-0.1 ppm).$[\alpha]^{20}_D$: +35.0 (*c*1.0, chloroform).

4.2. Biological Evaluation. Cell Culture and Transfection.

Cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell culture media, foetal bovine serum (FBS), and additives were provided by Invitrogen. CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 2mM L-glutamine, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Nonessential amino acids (Invitrogen) were also added to the media.

Transient transfection of the cell lines was performed using electroporation in a 300µL volume with a total of 10 µg of DNA (pRK5 Neo-NK1 wild type) plasmid up to 500ng plus pRK5 as carrier DNA to reach 10µg) containing 10^7 cells in electroporation buffer (50mM K_2HPO_4, 20mM CH_3COOK, 20mM KOH, and 26mM $MgSO_4$, pH 7.4). After electroporation (280 V, 1mF, GeneZapper 450/2500; IBI, New Haven, CT), cells were suspended in complete medium and seeded into 96-well culture plates at a density of 10^5 cells per well. 96-well culture plates were first coated with polyornithine diluted in PBS, incubated at 37°C for 30 min, and then rinsed with PBS before seeding.

4.2.1. *ELISA*.

To measure the expression of the transfected receptors, cells were transfected with pRK5-NK1-6His. 24 hours after electroporation, cells were fixed with 4% paraformaldehyde in PBS for 5 min and rinsed three times with PBS. A blocking step of 30 min with PBS +1% decomplemented FBS was performed before incubation with an anti-6 His primary antibody (0.5 µg/ml) for 30 min. The cells were then rinsed four times for 5 min in PBS +1% FBS and incubated for 30 min with an anti-mouse antibody conjugated with horseradish peroxidase (1/1000; Amersham, Orsay, France). The cells were rinsed three times with PBS+1% FBS and three times with PBS. Afterward, 60 µl of PBS and 20 µl of Supersignal ELISA Femto (Perbio-Pierce, Brebières, France) were added to the wells. The luminescence was read using a Wallac Victor2 (PerkinElmer Life and Analytical Sciences, Courtaboeuf, France)

4.2.2. Second Messenger (IP_1) Accumulation. Activation/inhibition of the IP pathway by NK1 receptor agonists or antagonists, respectively, was determined using the IP-One dynamic kit (Cisbio Bioassays, Bagnols-sur-Cèze, France). In brief, after transfection, 10^5 cells were distributed in 100 µl of complete medium into a 96-well assay plate (Greiner Bio-One, Courtaboeuf, France). Twenty-four hours later, the medium was removed and replaced with 40 µl of incubation medium containing the agonist and/or antagonist at the appropriate concentrations. The IP-One assay is based on the accumulation of IP_1, a downstream metabolite of the IP pathway that is produced by phospholipase C activated by the Gq/11 protein; IP_1 is stable in the presence of LiCl. The homogeneous time-resolved fluorescence-fluorescence resonance energy[42]transfer (HTRF-FRET) assay was performed as described previously. This assay involves the transfer of energy from a Lumi4^{TM}-Terbium cryptate donor fluorophore to a

d2 acceptor fluorophore. The assay is an immunoassay that measures competition between native IP_1 produced by the cells and IP_1 labelled with the d2 acceptor, as revealed by a monoclonal antibody against IP_1 labelled with Lumi4^{TM}-Terbium cryptate. Fifteen microlitres of antibody and 15 µl of competitor diluted in lysis buffer provided in the kits were added to the wells after 30-min incubation at 37 °C with the agonist. As a negative control, some wells only received the donor fluorophore-labeled antibody. After 1 h incubation at room temperature, fluorescence emissions were measured at both 620 and 665 nm on a RubyStar fluorometer (BMG Labtechnologies, Offenburg, Germany) equipped with a nitrogen laser as the excitation source (337 nm). A 400-µs reading was recorded after a 50-µs delay to eliminate the short-lived fluorescence background from the acceptor fluorophore-labeled antibody. The fluorescence intensities measured at 620 nm and 665 nm correspond to the total europium cryptate emission and to the FRET signal, respectively. The specific FRET signal was calculated using the following equation:

$\Delta F\% = 100x(Rpos - Rneg)/(Rneg)$, with Rpos being the fluorescence ratio (665/620 nm) calculated in wells incubated with both donor- and acceptor-labelled antibodies, and Rneg being the same ratio for the negative control incubated only with the donor fluorophore-labelled antibody. The FRET signal ($\Delta F\%$), which is inversely proportional to the concentration of IP_1 in the cells, was then transformed into IP_1 accumulation using a calibration curve prepared on the same plate. It is worth noting that all comparisons of agonist or antagonist effects were done on the same day, on the same culture and plate, and were made against the SP effect. The experiments were repeated at least three times on different cultures. Normalization was performed as indicated in the figure legends, either as a percentage of the maximal value or as a percentage of the maximal value for SP and CP-96345 when comparisons were necessary.

Values corresponding to the low basal activities, determined in unstimulated cells, were first subtracted. Activation/inhibition curves were plotted to the log of agonist or antagonist concentrations and fitted to the Hill equation to extract the EC50, Hill coefficient, and minimal/maximal values (see Figure 1, SI)

The inhibitory effect of the specific nonpeptidic NK1 antagonist on IP_1 accumulations induced by SP was studied according to Arunlakshana and Schild.[43]Preincubation for 10 min with the antagonist was followed by 30-min incubation with the antagonist and SP. IP_1 accumulation was then measured as described above.

*4.3.*Cytotoxicity Assay.

The human lung adenocarcinoma A549 cell line and the human embryo lung fibroblastic MRC-5 cell line were obtained from the European Collection of Cell Cultures. They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% foetal bovine serum (FBS), and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37º C.

Cell proliferation was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A total of $9x10^3$ cells/well (MRC-5 cells) and $4,5x10^3$ cells/well (A549 cells) were cultured in 96-well plate for 24 h. Drugs were then added to the plates. After 48 hours, medium was removed and 125 µl MTT (1 mg/ml in medium) were added for 4 hours. Then, 80 µl 20% SDS in 0,02 M HCl were added and incubated for 10 hours at 37 ºC. The optical density (OD) of each well was measured at 540 nm with a multiwell plate spectrophotometer reader to quantify cell viability. Results were expressed as percentage of cell viability in relation to controls.

4.4.Statistical Analysis.

Statistical significance of the differences between experimental groups was determined by one-way or two-way analysis of variance followed by a post hoc Duncan's multiple range test to make pairwise comparisons between means. All data were averaged from at least three independent experiments and were expressed as means ± standard deviation of the means (SEM). For statistical analysis we used the t-test (paired, two-tailed); a P-value >0.05 is not considered statistically significant and is not represented by any symbol, a P-value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a P-value <0.01 is indicated with 2 asterisks, and a P-value <0.001 is indicated with 3 asterisks.

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ABBREVIATIONS

DMSO, dimethyl disulfide;FRET, fluorescence resonance energy; GPCR, G-protein coupled receptor; HTRF, Homogeneous Time-Resolved Fluorescence; NK1R, neurokinin 1 receptor; NMR, nuclear magnetic resonance spectroscopy; SD, standard deviation.

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GRAPHICAL ABSTRACT.