UNIVERSIDAD COMPLUTENSE DE MADRID
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TESIS DOCTORAL

New molecules inspired on microbiota metabolites targeting cancer phenotypes

Nuevas moléculas inspiradas en metabolitos de la microbiota y su estudio fenotípico en cáncer

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Departamento de Química Orgánica

NEW MOLECULES INSPIRED ON MICROBIOTA METABOLITES TARGETING CANCER PHENOTYPES

NUEVAS MOLÉCULAS INSPIRADAS EN METABOLITOS DE LA MICROBIOTA Y SU ESTUDIO FENOTÍPICO EN CÁNCER

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NEW MOLECULES INSPIRED ON MICROBIOTA METABOLITES TARGETING CANCER PHENOTYPES

NUEVAS MOLÉCULAS INSPIRADAS EN METABOLITOS DE LA MICROBIOTA Y SU ESTUDIO FENOTÍPICO EN CÁNCER

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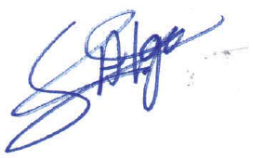

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A Alejandro y Esperanza;
siempre estaréis acompañándome.

El presente trabajo ha sido realizado en el Laboratorio de Química Médica del Departamento de Química Orgánica de la Facultad de Ciencias Químicas de la Universidad Complutense de Madrid, bajo la supervisión de la ***Catedrática María Luz López Rodríguez****, la* ***Prof. Bellinda Benhamú Salama*** *y la* ***Prof. María del Henar Vázquez Villa*** *a quienes deseo expresar mi afecto y mi más sincero agradecimiento por su calurosa acogida en este grupo de investigación, por sus continuas enseñanzas a lo largo de estos años, y muy especialmente, por todo el ánimo, apoyo y confianza depositados en mí para la realización de este proyecto.*

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ABBREVATIONS AND ACRONYMS

Throughout this manuscript, abbreviations and acronyms recommended by the American Chemical Society in the Organic Chemistry and Medicinal Chemistry areas have been employed (revised in the *Journal of Organic Chemistry* and *Journal of Medicinal Chemistry* on December 2018; https://pubs.acs.org/paragonplus/submission/joceah/joceah_abbreviations.pdf and https://pubs.acs.org/paragonplus/submission/jmcmar/jmcmar_abbreviations.pdf). In addition, those indicated below have also been used:

ACN	Acetonitrile
AcOH	Acetic acid
APC	Allophycocyanin
app	Apparent
ATCC	American type culture collection
bFGF	Basic fibroblastic growth factor
CAI	Centro de apoyo a la investigación
CFU	Colony-forming unit
cpr	Cyclopropane
CSC	Cancer stem cell
DIPEA	*N*,*N*-Diisopropylethylamine
DMEM	Dulbecco's modified eagle medium
ECACC	European collection of authenticated cell cultures
EDC	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
HOBt	1-Hydroxybenzotriazole
IL-3	Interleukin-3
iTRAQ	Isobaric tags for relative and absolute quantification
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide

MW	Microwave
PDD	Phenotypic drug discovery
PerCP	Peridinin chlorophyll protein
PNBA	*p*-Nitrobenzoic acid
PolyHEMA	Poly(2-hydroxyethyl methacrylate)
PRG	Peptidic reactive group
PrSc	Privileged scaffolds
SCF	Stem cell factor
TDD	Target-based drug discovery
UCM	Universidad Complutense de Madrid
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

RESUMEN

NUEVAS MOLÉCULAS INSPIRADAS EN METABOLITOS DE LA MICROBIOTA Y SU ESTUDIO FENOTÍPICO EN CÁNCER

En las últimas décadas, el descubrimiento de fármacos dirigidos a una diana terapéutica específica, *target-based drug discovery* (TDD), ha sido la estrategia predominante en la búsqueda de nuevos fármacos. Sin embargo, recientemente ha resurgido el interés por los programas basados en estudios fenotípicos, *phenotypic drug dicovery* (PDD), los cuales permiten identificar compuestos que inducen una respuesta biológica de interés sin la necesidad de conocer la diana molecular subyacente. Los ensayos fenotípicos proporcionan un enfoque menos sesgado para detectar moléculas capaces de regular una diana terapéutica y los compuestos identificados suelen tener un mayor impacto terapéutico *in vivo*. En este contexto, el presente proyecto de química médica se ha abordado mediante un programa de PDD enfocado al descubrimiento de nuevos fármacos antitumorales.

La microbiota humana es un complejo ecosistema de microorganismos simbiontes que juega un papel fundamental en la salud del ser humano, y en el cual la interacción con el huésped se debe en parte a la secreción de metabolitos que pueden regular proteínas humanas. Estudios recientes sugieren la influencia de la microbiota y sus metabolitos en diferentes patologías como el cáncer, la obesidad, la diabetes, desórdenes en el sistema inmune o enfermedades neurológicas. Concretamente, en el contexto del cáncer, diversos metabolitos han demostrado tener un papel importante en la protección tumoral o como coadyuvantes en tratamientos de quimioterapia y radioterapia. Por tanto, los metabolitos producidos por la microbiota podrían constituir una fuente de nuevas moléculas con propiedades biológicas interesantes. En este sentido, en este trabajo hemos desarrollado nuevas moléculas basadas en metabolitos de la microbiota que podrían conducir a nuevas terapias para el tratamiento del cáncer.

El cáncer representa la segunda causa de muerte por enfermedad en el mundo. A pesar del diagnóstico precoz y el desarrollo de nuevas terapias, en muchos pacientes se produce un fracaso terapéutico, lo que da lugar a la progresión de la enfermedad, la aparición de recidivas, y la reducción de la supervivencia. Una de las razones de la reaparición de un tumor es la heterogeneidad intra-tumoral debido a la existencia de diferentes tipos de células dentro de un mismo tumor, entre las que se han identificado células madre de cáncer o *cancer stem cells* (CSCs). Estas células son fundamentales en la iniciación y progresión de muchos tumores, y al tratarse de células relativamente quiescentes, no resultan afectadas por las terapias convencionales diseñadas para eliminar células diferenciadas altamente proliferativas. Por tanto, aquellos tratamientos que sean capaces de actuar frente a la población de CSCs presente en un tumor, bien provocando su muerte o induciendo su diferenciación, ofrecen una nueva estrategia para curar eficazmente la enfermedad, pues conducirían a una población remanente de células tumorales diferenciadas que podrían ser eliminadas mediante las terapias convencionales (Figura 1).

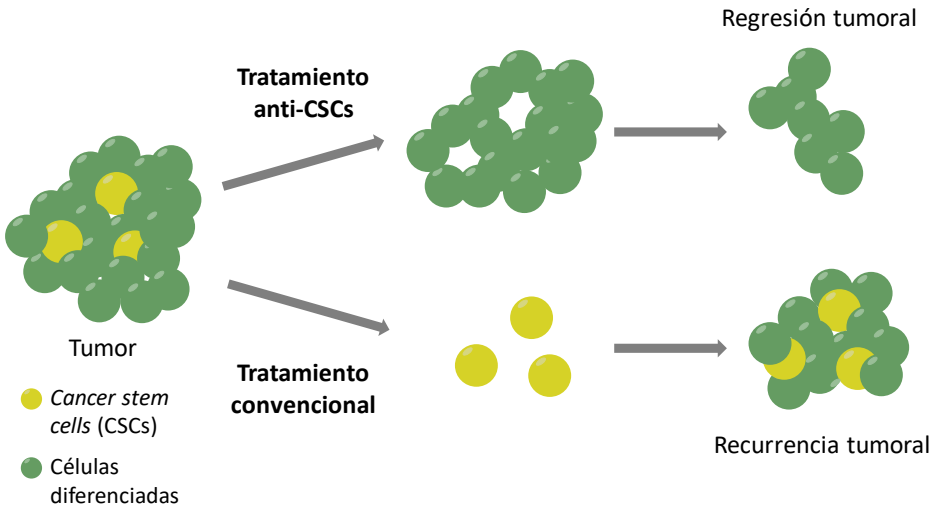


Figura 1. Terapias dirigidas a CSCs para el tratamiento del cáncer

En este contexto, el presente trabajo de investigación está dirigido al desarrollo de nuevos compuestos basados en los metabolitos de la microbiota humana que sean capaces de actuar sobre las CSCs. Con este objetivo, se diseñaron nuevos compuestos considerando estructuras privilegiadas (PrSc) presentes en los metabolitos y se plantearon las rutas sintéticas correspondientes, empleando como primera etapa de la síntesis reacciones organocatalíticas

asimétricas, con el fin de generar diversidad estructural de forma eficaz y estereocontrolada. La estructura general de los compuestos diseñados **1a,b**-**20a,b** consta de un esqueleto central basado en PrSc al cual se han incorporado otras PrSc y un fragmento considerado *drug-like* que pueda contribuir a unas buenas propiedades farmacocinéticas (Figura 2). El primer paso de la ruta sintética propuesta consistió en la reacción entre un aldehído α,β-insaturado y un nitrocompuesto, catalizada por un derivado enantiopuro del prolinol, obteniéndose estructuras diferentes con una gran diastereo- y enantioselectividad. A continuación, y teniendo en cuenta que el objetivo final es desarrollar candidatos a fármacos, se llevó a cabo la derivatización de los productos resultantes mediante la reducción del grupo nitro a amina y la transformación del aldehído en el fragmento *drug-like* (ciclopropilmetil)amina (Figura 2).

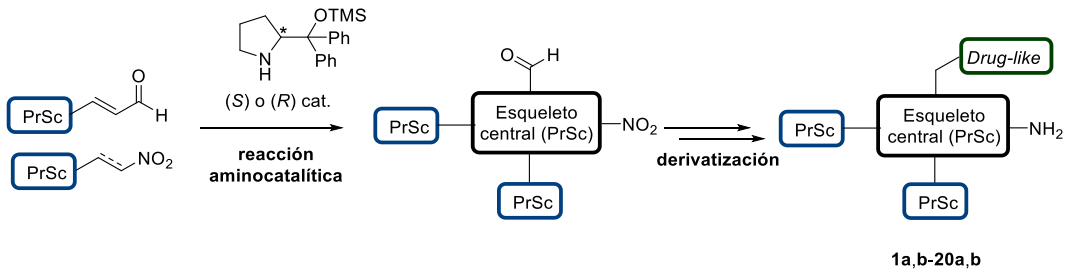


Figura 2. Ruta sintética empleada para la obtención de los compuestos **1a**,**b**-**20a,b** basados en los metabolitos de la microbiota humana

Los compuestos sintetizados fueron estudiados en ensayos fenotípicos celulares con el objetivo de determinar su capacidad para regular procesos de diferenciación celular en modelos de cáncer. Se comenzó evaluando la citotoxicidad de los compuestos en la línea tumoral de mama (MCF-7) y de colon (HCT-116) a diferentes concentraciones (10, 5, 1, 0.5, 0.1 µM), empleando un ensayo colorimétrico MTT. A continuación, se estudió el proceso de formación de esferas, un crecimiento característico de las CSCs, incubando durante 14 días las mismas líneas tumorales, en presencia de la concentración no tóxica del compuesto determinada en el ensayo de citotoxicidad. Los compuestos que en este ensayo fueron capaces de inhibir la formación de mamo-esferas y colono-esferas en más de un 90% respecto a las células no tratadas, sin presentar toxicidad en células tumorales diferenciadas, se caracterizaron como compuestos que actúan en CSCs promoviendo su diferenciación y/o induciendo su muerte. Adicionalmente, se evaluó la citotoxicidad en la línea de fibroblastos IMR90 para

determinar si los compuestos afectan la viabilidad de las células no tumorales. Teniendo en cuenta el conjunto de los resultados del *screening* fenotípico, se seleccionaron los compuestos **4b** (UCM13369) y **14b** (UCM13218), que mostraron una inhibición completa de la formación de esferas y no eran citotóxicos en líneas tumorales ni en fibroblastos, para estudiar su potencial terapéutico en muestras de sangre de pacientes de leucemia mieloide aguda (LMA), como modelo *ex vivo* de CSCs.

Las células madre hematopoyéticas (células $CD34^+$) se aislaron a partir de muestras de médula ósea y se emplearon para realizar ensayos de formación de colonias (CFU). Como se muestra en la Figura 3 (barras negras), los compuestos disminuyeron significativamente la formación de colonias derivadas de células $CD34^+$ de LMA en el rango µM y de manera dosis-dependiente (CI_{50} < 5 µM). Estos resultados indican que los compuestos son capaces de inducir la muerte de las células madre $CD34^+$ y/o de promover su diferenciación. Además, se observó una disminución en el número de células viables en presencia de ambos compuestos (Figura 3, barras grises), lo que corrobora su efecto en la proliferación de células hematopoyéticas de LMA.

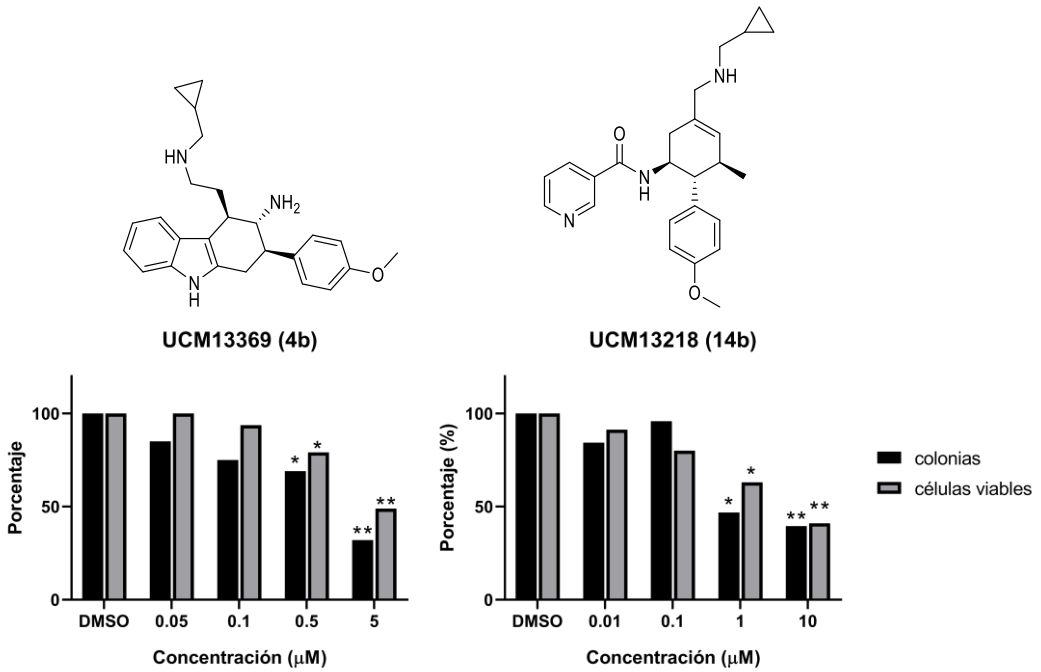


Figura 3. Ensayo de formación de colonias (CFU) en células CD34⁺ de LMA. El número de colonias y células viables se determinó usando un microscopio de contraste de fase en ausencia o presencia de los compuestos UCM13369 (**4b**) y UCM13218 (**14b**). Todos los ensayos fueron realizados por duplicado ($p<0.05$ *; <0.01 **)

A continuación, se analizó la población de células derivadas de las colonias mediante citometría de flujo, utilizando los correspondientes anticuerpos de CD34 como marcador de *stemness*, CD71 como marcador de eritroblastos (precursores de eritrocitos) y CD45 como marcador de leucocitos. Además, se empleó anexina V para determinar la población celular apoptótica. En las muestras de pacientes de LMA se puede observar una gran población de células inmaduras CD71⁺ y una baja población de células diferenciadas CD45⁺ (Figura 4A), característico de pacientes con LMA. Esta proporción se mantuvo cuando tratamos las células con UCM13218, mientras que tras la administración de UCM13369 la proporción de células CD71⁺ disminuyó y la de células CD45⁺ aumentó. Por otro lado, ambos compuestos incrementaron la apoptosis de células inmaduras CD71⁺ (Figura 4B), sin afectar a las células diferenciadas CD45⁺ (Figura 4C). Estos resultados indican que UCM13218 y UCM13369 inducen la muerte de células progenitoras (CD71⁺), no así la de células sanguíneas diferenciadas (CD45⁺). Además, UCM13369 promueve la diferenciación mieloide de células madre hematopoyéticas (CD34⁺)

a leucocitos ($CD45^+$), lo que confirma que ambos compuestos pueden regular el proceso de diferenciación y/o la muerte en las células madre de LMA.

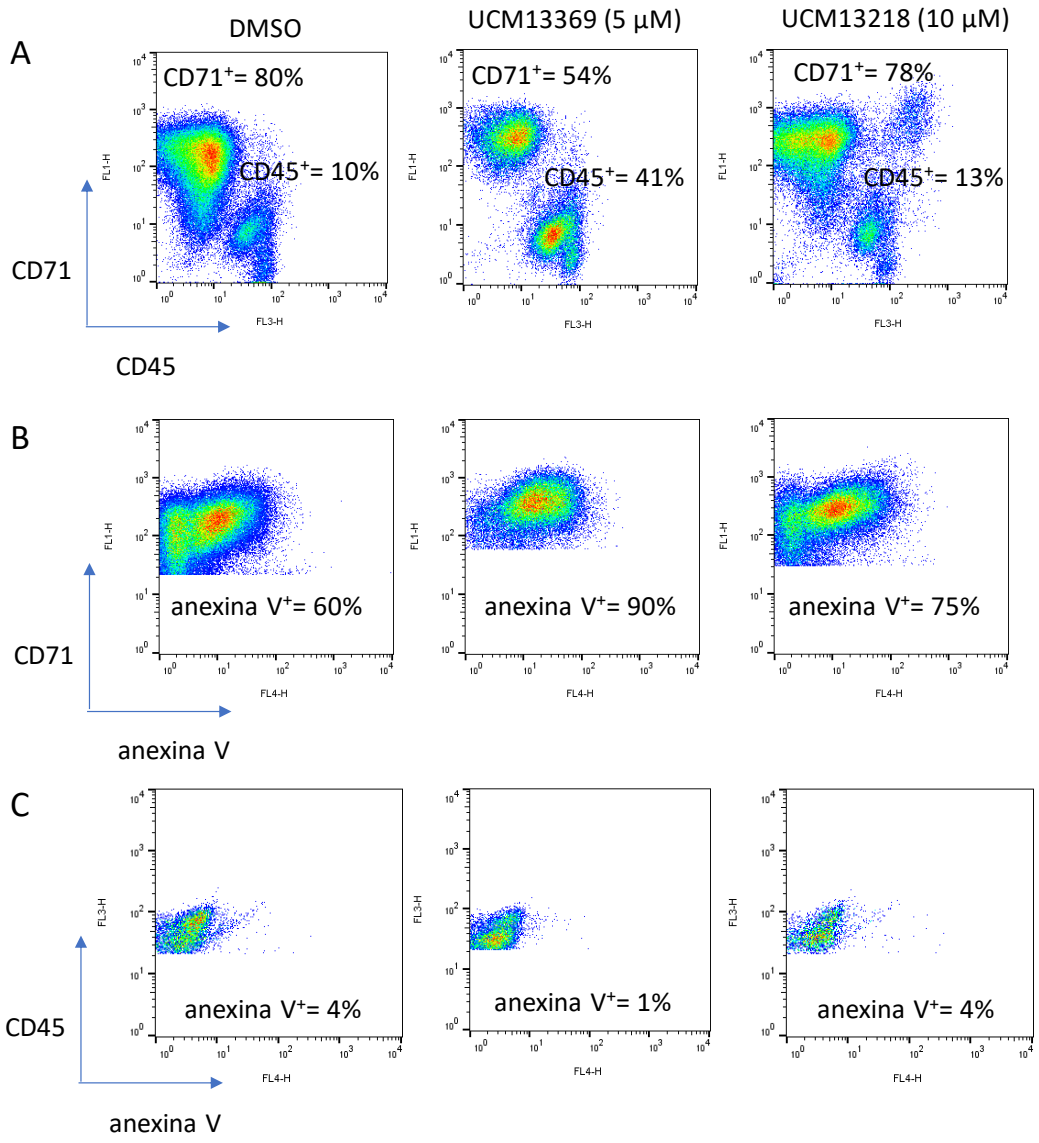


Figura 4. Análisis de citometría de flujo de las poblaciones $CD71^+$-$CD45^+$ (A), $CD71^+$-anexina V^+ (B), y $CD45^+$-anexina V^+ (C) después de 14 días de tratamiento de células $CD34^+$ de LMA con UCM13369 (5 µM) y UCM13218 (10 µM)

En la actualidad, se están realizando experimentos de proteómica diferencial (iTRAQ) en muestras de células $CD34^+$ para identificar aquellas proteínas cuyos niveles de expresión se modifican tras el tratamiento con los compuestos, lo que ayudará a estudiar el mecanismo de acción de los nuevos compuestos UCM13369 y UCM13218. Por otro lado, se ha planificado su evaluación *in vivo* en un modelo de xenoinjerto humano de LMA en ratones inmunodeprimidos, con el objetivo de confirmar el potencial terapéutico en leucemia de los nuevos compuestos identificados en este trabajo.

SUMMARY

NEW MOLECULES INSPIRED ON MICROBIOTA METABOLITES TARGETING CANCER PHENOTYPES

In the past three decades, target-based drug discovery (TDD) has been the dominant approach to drug discovery. However, in recent years, there has been a renewed interest in phenotypic drug discovery (PDD) approaches, which allow to identify biologically active molecules in a physiologically relevant system, without needing to know the target protein a priori. In the present medicinal chemistry project, a PDD program will be addressed to identify new drug candidates.

Human microbiota is a complex ecosystem of symbiotic microorganisms that play an important role in human health and disease. The crosstalk between the host and its microbiome occurs in part through the secretion of metabolites, which can regulate human proteins. Recent studies suggest the influence of the microbiota and its metabolites in several diseases, such as cancer, obesity, diabetes, immune system disorders, or neurological pathologies. In the context of cancer, various microbiota metabolites have been shown to play a major role in tumor protection. Therefore, these metabolites could represent an unexplored chemical space with important biological activities. In this work, we have developed new small molecules inspired on microbiota metabolites that could lead to novel therapeutic strategies for cancer treatment.

Regardless of early diagnosis and the development of new treatments, many cancer patients still fail therapy, causing the tumor relapse. One of the reasons is the enormous intra-tumor heterogeneity in the tumor cells. Among them, cancer stem cells (CSCs) are usually more resistant to conventional treatments leading to tumor recurrence. Therefore, a promising therapeutic strategy for cancer and metastatic processes is to induce CSC death or differentiation (Figure 1).

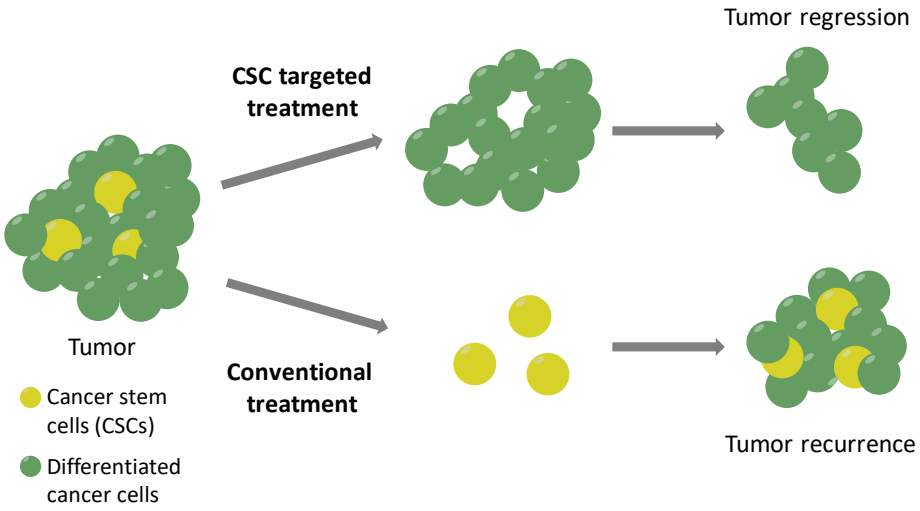


Figure 1. CSC targeted therapies for cancer treatment

In this context, the present research project is focused on the development of new compounds based on microbiota metabolites that are able to act on CSCs. For this purpose, we first designed new compounds considering privileged scaffolds (PrSC) present in microbiota metabolites and taking advantage of an asymmetric organocatalytic reaction as a key synthetic step to generate structural diversity with high efficiency and in stereocontrolled manner. The general structure of the designed molecules **1a,b**-**20a,b** consists of a PrSc-based central core to which other PrSc are linked, and a drug-like fragment to improve the pharmacokinetic properties (Figure 2). The synthesis started with an aminocatalytic reaction between an α,β-unsaturated aldehyde and a nitrocompound, using an enantiopure prolinol derivative as catalyst, to afford structurally different scaffolds with high diastereo- and enantioselectivity. The resulting products were further derivatized: the nitro group was reduced to amino, and the aldehyde transformed into a (cyclopropylmethyl)amine as the drug-like fragment.

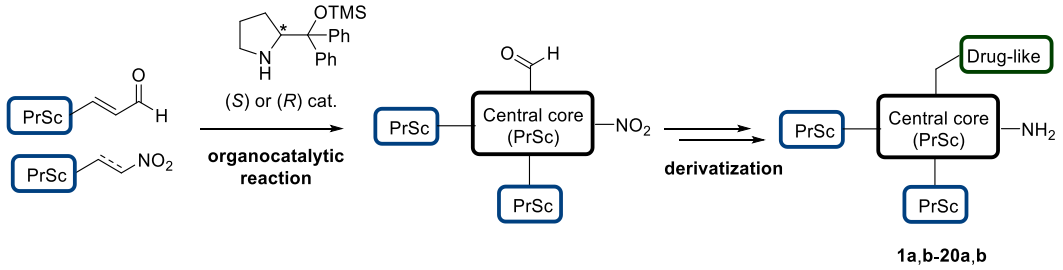


Figure 2. Synthetic pathway to obtain new microbiota-inspired molecules **1a**,**b**-**20a**,**b**

New synthesized compounds were screened in cellular phenotypic assays to evaluate their ability to regulate the differentiation process in cancer models. First, the cytotoxicity in breast MCF-7 and colon HCT-116 cancer cell lines was evaluated at different concentrations of the new compounds (10, 5, 1, 0.5, 0.1 µM), using a colorimetric MTT assay. The resulting non-toxic concentration was then used to study the tumorsphere formation, as a characteristic growth in CSCs, after incubation with the compounds during 14 days. Those compounds able to inhibit the formation of mammospheres and colonspheres (inhibition >90% relative to non-treated cells), with no cytotoxicity in the corresponding non-stem cells, were considered to act on CSCs by promoting differentiation and/or inducing their death. Next, MTT cytotoxicity assay was performed in the fibroblast IMR90 cell line in order to determine if the compounds are able to affect the viability of non-tumor cells. From the results obtained in the cellular phenotypic screening, compounds **4b** (UCM13369) and **14b** (UCM13218), exhibiting inhibition of tumorsphere formation and no cytotoxicity in tumor and non-tumor cells, were selected to test their therapeutic potential in blood samples of acute myeloid leukemia (AML) patients as an *ex-vivo* CSC model.

$CD34^+$ hematopoietic stem cells were isolated from bone marrow and then used to perform colony-forming unit (CFU) assays. As shown in Figure 3 (black bars), the compounds significantly decreased the formation of colonies derived from AML $CD34^+$ cells in the µM range and in a dose-dependent manner (IC_{50} < 5 µM). This result indicates that UCM13369 and UCM13218 induce the death of stem $CD34^+$ cells and/or promote the differentiation to blood cells. In addition, a decrease in the number of viable cells was observed in the presence of both compounds (Figure 3, grey bars), which supports their effect in the proliferation of AML hematopoietic cells.

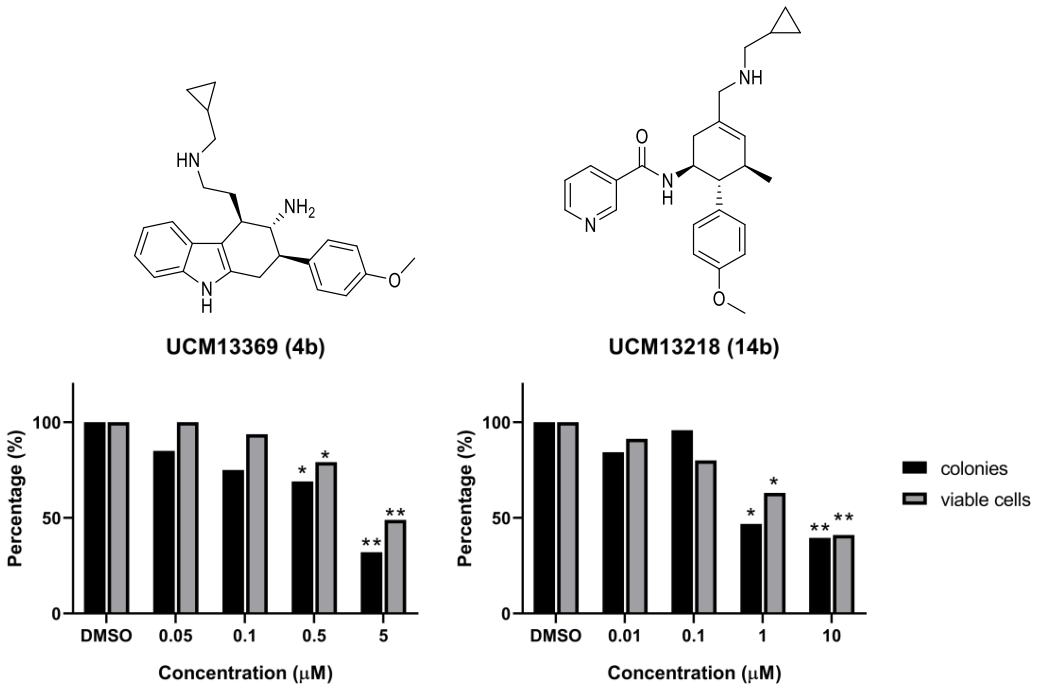


Figure 3. Colony-forming unit (CFU) assay in AML CD34$^+$ cells. The number of colonies and viable cells were determined using phase contrast microscopy in the absence or presence of selected compounds UCM13369 (**4b**) and UCM13218 (**14b**). All assays were performed in duplicate ($p<0.05$ *; <0.01 **)

Next, the cell population from colonies was analysed by flow cytometry using CD34 antibody as stemness marker, CD71 as erythroblast (precursor of erythrocyte) marker, and CD45 as leukocyte marker. Also, annexin V was employed to determine the cellular apoptotic population. As AML is characterized by the accumulation of progenitor myeloblasts, patient samples display a higher CD71$^+$ erythroblast population than differentiated CD45$^+$ leukocytes (Figure 4A). This ratio was maintained in the presence of compound UCM13218, while after the administration of analogue UCM13369, CD71$^+$ population decreased and CD45$^+$ population increased. On the other hand, both compounds increased the apoptosis of unmature CD71$^+$ cells (Figure 4B). Notably, differentiated CD45$^+$ cells were not affected by treatment (Figure 4C). Hence, UCM13218 and UCM13369 induce cell death of unmatured (CD71$^+$) cells but not differentiated cell (CD45$^+$) death. UCM13369 also promotes stem cell (CD34$^+$) differentiation to leukocytes (CD45$^+$). These results confirm that both compounds are able to regulate the differentiation process and/or death in AML.

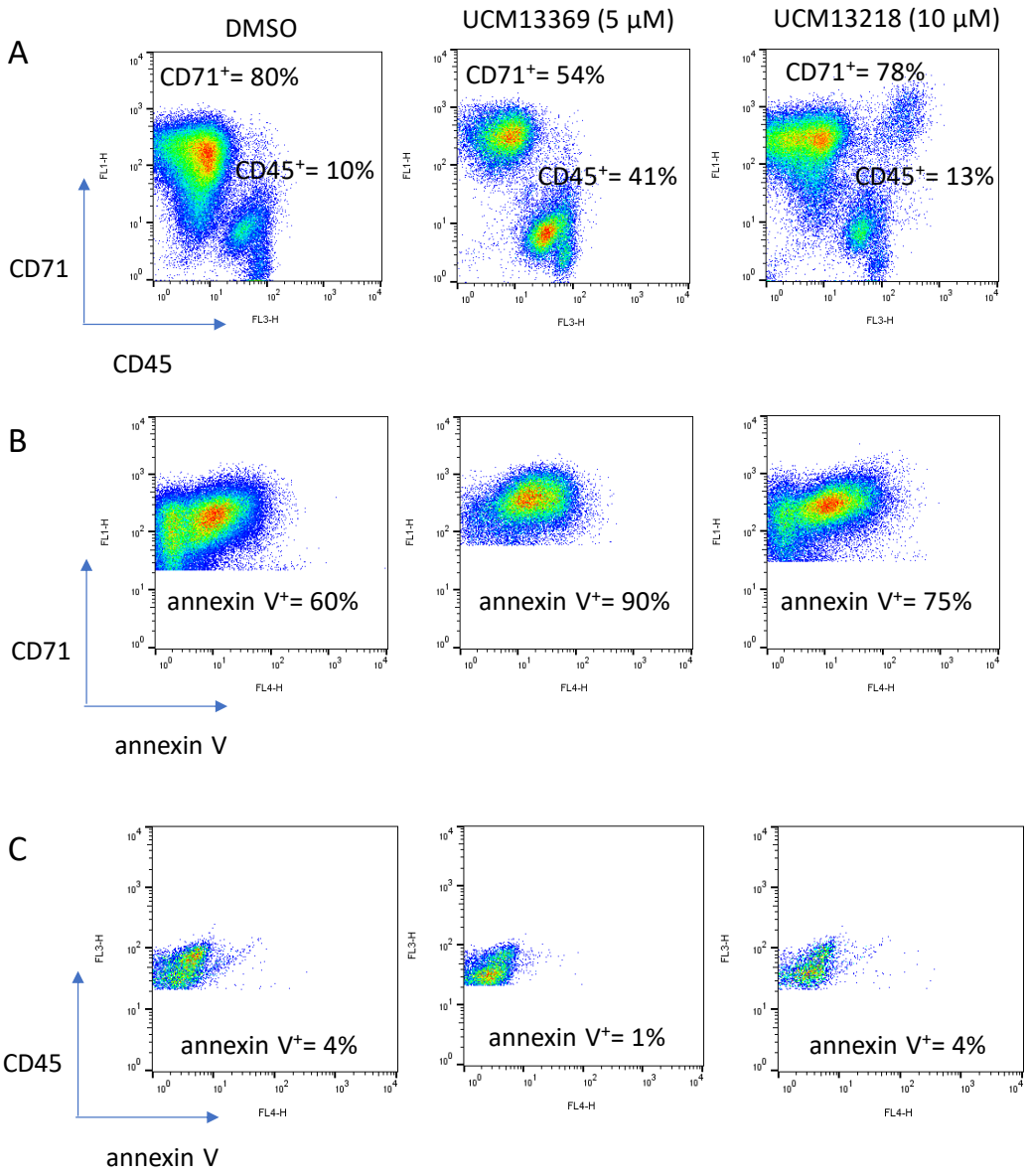


Figure 4. Flow cytometry analysis of CD71$^+$-CD45$^+$ (A), CD71$^+$-annexin V$^+$ (B), and CD45$^+$-annexin V$^+$ (C) populations after 14 days of treatment of AML CD34$^+$ cells with UCM13369 (5 µM) and UCM13218 (10 µM)

Currently, differential proteomic experiments (iTRAQ) are in course in CD34$^+$ samples to quantify the expression levels of the proteins regulated by the new compounds. The identification of the drug target protein(s) will allow to study the mechanism of action. Additionally, *in-vivo* evaluation of UCM13369 and UCM13218 is under study in a human AML xenograft in immunodeficient mice, to confirm their therapeutic potential in leukemia.

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION AND OBJECTIVES

In the past three decades, target-based drug discovery (TDD) –in which the starting point is a defined molecular target that is hypothesized to have an important role in disease– has been the dominant approach to drug discovery. However, in recent years, there has been a renewed interest in phenotypic drug discovery (PDD) approaches, which do not rely on knowledge of the identity of a specific drug target or a hypothesis about its role in disease. In the case of PDD, a physiologically relevant biological system or cellular signalling biological pathway is directly interrogated by chemical compounds to identify biologically active molecules. In this primary screening assay, hits in the cellular phenotype model may reveal different molecular phenotypes corresponding to different mechanisms of action (MoAs). MoAs that affect disease-relevant pathways will be evaluated for in vivo proof-of-concept. Nonspecific MoAs can be eliminated using molecular phenotype information (Figure 1).[1]

PDD is a challenging strategy based on its potential to address the incompletely understood complexity of disease and its promise of delivering first-in-class drugs. It is also a powerful approach to exploit the novel biological space of undrugged or unknown targets, providing a route to enhance innovation and to deliver truly novel therapeutics for unmet medical needs. In the present medicinal chemistry project, a PDD program will be addressed.

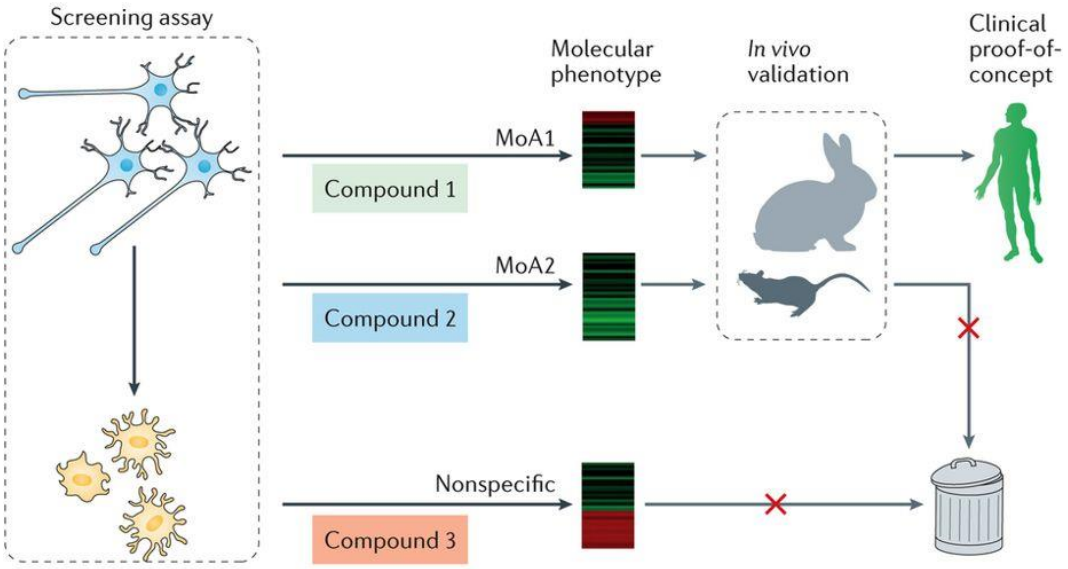


Figure 1. Phenotypic drug discovery (PDD) approach (source: reference 1)

1.1. Microbiota in health and disease

Humans are viewed as composites of human and microbial cells. Human microbiota is a complex ecosystem of trillion of symbiotic microorganisms composed of protozoa, fungi, virus and bacterial species that play an important role in human health and disease. Microbial cells have their own genetic code, and the set of genes these cells hold are known as microbiome. While human genome consists of about 23000 genes, microbiome encodes over three million genes, providing unique and specific enzymes and biochemical pathways to the host.[2]

The composition of the microbiota differs from person to person and between different parts of the same person. In fact, differences in the microbiome composition can help explain why some people are more susceptible or resistant to certain diseases.[3] Diet, environment, host genetics, and early microbial exposure seem to be implicated in this diversity.[4]

Human microbiota has beneficial functions to the host that are key for maintaining homeostasis. Microbiota acts as a physical barrier, protecting host from external pathogens. In addition, these microorganisms are fundamental in the development of the immune system of the host. Moreover, the microbiota can synthesize, modulate and degrade a large repertoire of small molecules, providing a functional complementation to the metabolic capacities of the host. In particular, the microbiota contributes to the absorption of nutrients as they metabolize dietary

components that cannot be metabolized by the host, such as complex carbohydrates, vitamins, neuroactive metabolites or amino acids.[5] These biochemical reactions significantly contribute to many aspects of the host's health, including metabolism, immunity, development, and behaviour.

Alterations in the composition of microbiota can result from exposure to environmental factors such as diet, xenobiotics, drugs, and pathogens. This microbial community imbalance, termed dysbiosis, is associated with disease since it eventually contributes to the pathogenesis of various metabolic, neurological, inmmunological, and oncogenic diseases (Figure 2).[6] It has been proved that if the microbiota of a diseased individual is replaced with a healthy one, the effect is positive and it improves the conditions of life.[7, 8]

Altogether, evidence has accumulated in the last decade that alterations of the microbiome are involved in the etiology of a multitude of human diseases, including cancer,[9-11] obesity,[12, 13] diabetes,[14-17] immune system disorders,[18-22] or neuropsychiatric pathologies.[23-26]

The crosstalk between the host and its microbiome occurs in part through the secretion of metabolites, which can regulate human proteins.[27] It has become increasingly apparent that microbiota metabolites are important orchestrators of host physiology through the control of a large range of metabolic, inflammatory, and even behavioural processes. For example, microbiota metabolites such as short chain fatty acids or tryptophan-metabolism products act as drivers of immune system development, differentiation, and activity.[28]

In the context of cancer, various microbiota metabolites have been shown to play a major role in tumor protection. For instance, phytoestrogens (e.g. urolithins and equol) bind to estrogen receptor to exert important protective effects against breast and prostate cancer.[29, 30] Recent studies have also shown that microbiota metabolites can act as a potent adjuvant for anticancer treatment such as chemotherapy, radiotherapy, and immunotherapy.[9]

Hence, microbiota metabolism produces a wide and diverse range of bioactive small molecules that are available to the host to regulate key biological functions. The compatibility of microbiota with healthy human systems suggests that metabolites represent drug-like molecules that are highly specific modulators and derisked for adverse effects.[30] This supports the hypothesis that microbiota metabolites include a unique repertoire of small organic molecules that represent an unexplored chemical space in the search of starting hits for medicinal chemistry programs towards the identification of novel drug candidates for the treatment of a wide range of pathological conditions.[29] In the present project, small molecules

inspired on human microbiota metabolites will be generated and explored in cancer phenotypes.

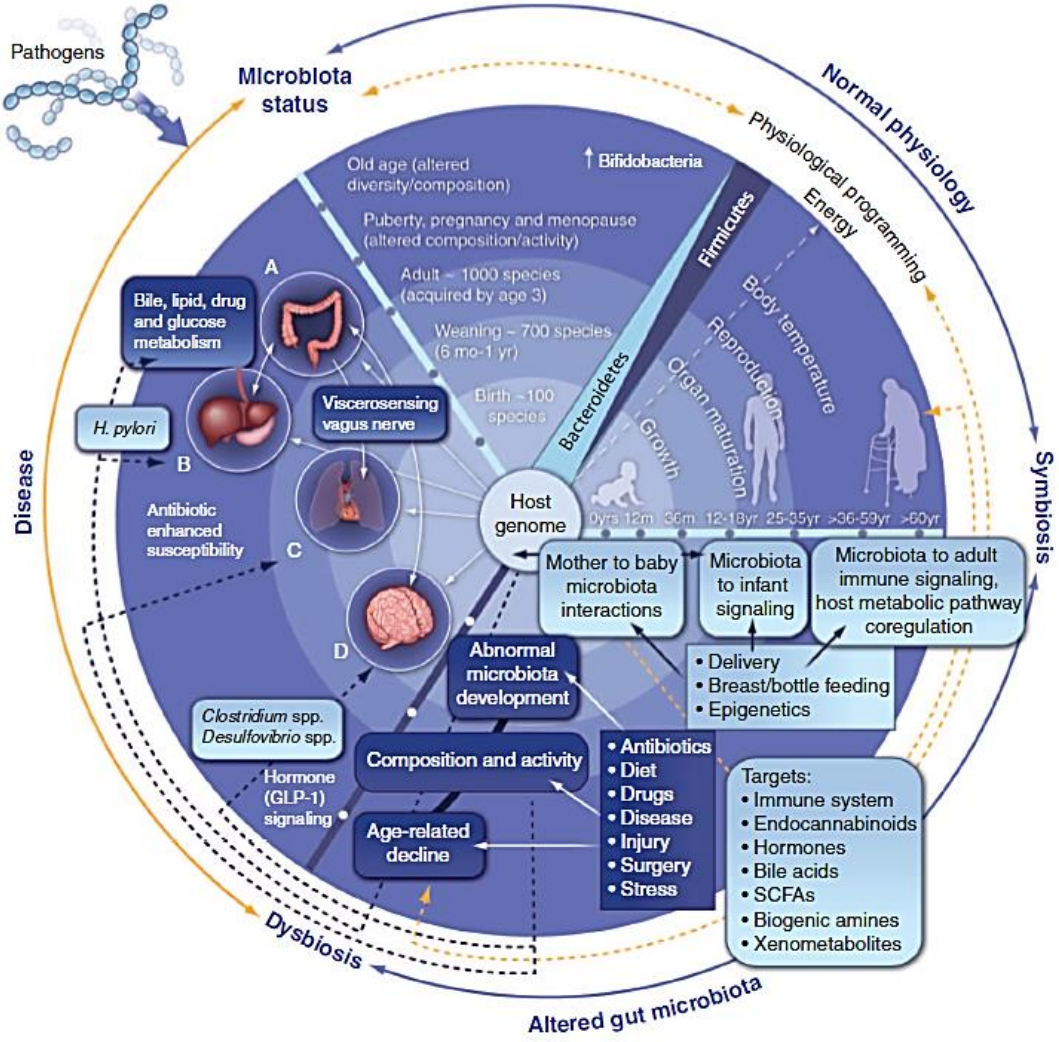


Figure 2. The human microbiota in health and disease (source: reference 6)

1.2. Targeting cancer stem cells (CSCs)

Cancer is defined as a set of diseases, in which the cells divide and grow abnormally and uncontrollably in a tissue or organ forming masses called tumors. Cancer is a major public health problem worldwide, which represents the second cause of death by disease in the world, and is responsible for estimated 9.6 million deaths in 2018; about 1 in 6 deaths is due to cancer according to World Health Organization.[31] Among the most common are lung (2.09 million cases), breast (2.09 million cases), and colorectal (1.80 million cases) cancers.

Cancer arises from the transformation of normal cells into tumor cells in a multistage process from pre-cancerous lesion to malignant tumor. These changes have both genetic and external components due to several factors: physical carcinogens (ultraviolet and ionizing radiation), chemical carcinogens (e.g. tobacco smoke, pollution), and biological carcinogens (infections from viruses, bacteria, or parasites).[32]

Tumor cells have specific characteristics such as the ability to maintain cell proliferation, to evade growth-suppressing mechanisms, to activate invasive processes and metastasis, to resist programmed death (apoptosis), to induce new blood vessels to supply oxygen and nutrients to tumours (angiogenesis), and to evade immune responses (Figure 3).[33, 34]

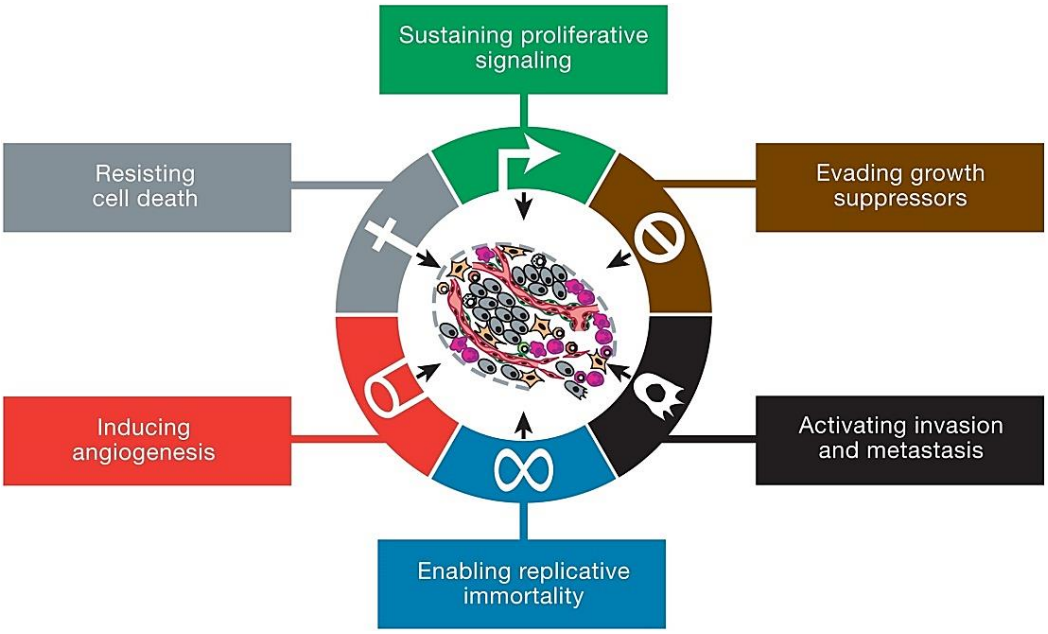


Figure 3. General characteristics of tumor cells. (source: reference 33)

Regardless of early diagnosis and the development of new treatments, many patients still fail therapy, causing the progression of the disease, relapse, and reduced overall survival. In most cancers, the mortality is due to the disease recurrence. One of the reasons is the enormous heterogeneity, not only inter-tumor or between patients, but also intra-tumor due to the existence of different cells within a tumor, from cancerous cells to normal cells, that contribute to its growth (Figure 4).[35-37]

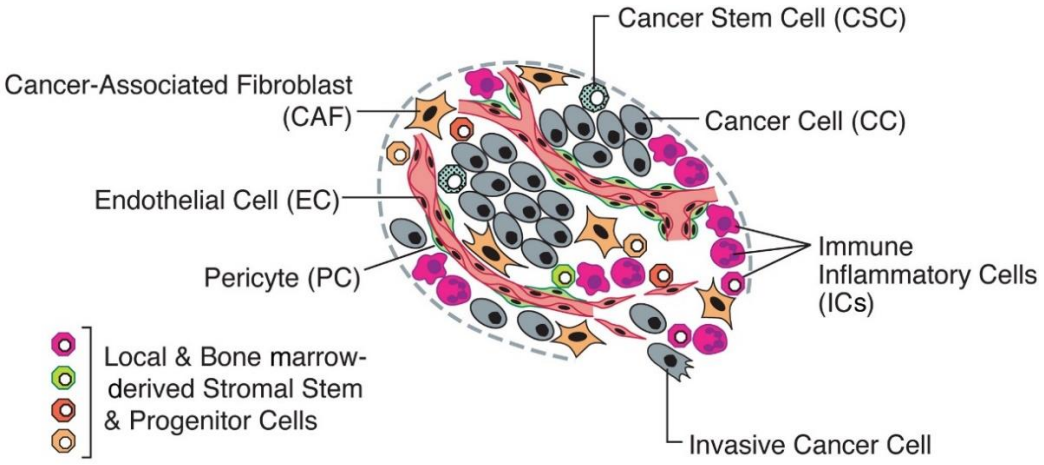


Figure 4. Cellular heterogeneity in a tumor mass (source: reference 33)

Several mechanisms have been studied to explain chemo- and radio-resistance to anti-tumor therapies. Interestingly, the presence of cancer stem cells (CSCs) has been related to therapy resistance and relapse. Moreover, recent studies suggest that CSCs are responsible for metastasis in several types of cancer.[38, 39]

For years, tumorigenesis and tumor progression have been explained by the stochastic model, in which cells become potentially tumorigenic when they acquire genetic mutations that allow them to proliferate in an uncontrolled manner. This model postulates all cancer cells have the capacity to generate a new tumor when these cells are transplanted into immunodeficient mouse. However, it has been demonstrated that one million of cells is necessary to form a new tumor, in contrast with the typical potential of cancer cells. On the other hand, the hierarchical model or CSC model postulates that tumors are composed of a heterogeneous cell population, being only CSCs, a small subpopulation of cancer cells, able to initiate

a primary tumor and metastatic cancer. This model explains that tumors have a hierarchical organization, being CSCs in the top of the hierarchy. These cells originate from healthy stem cells that undergo mutations. Successive asymmetric divisions of this type of cells will generate the rest of the tumor bulk with non-stem properties. The dynamic model is based on the hierarchical model and postulates that the stem-like phenotype can be acquired by non-stem cancerous cells, highlighting the dynamic nature of cancer cells and CSC heterogeneity (Figure 5).[40]

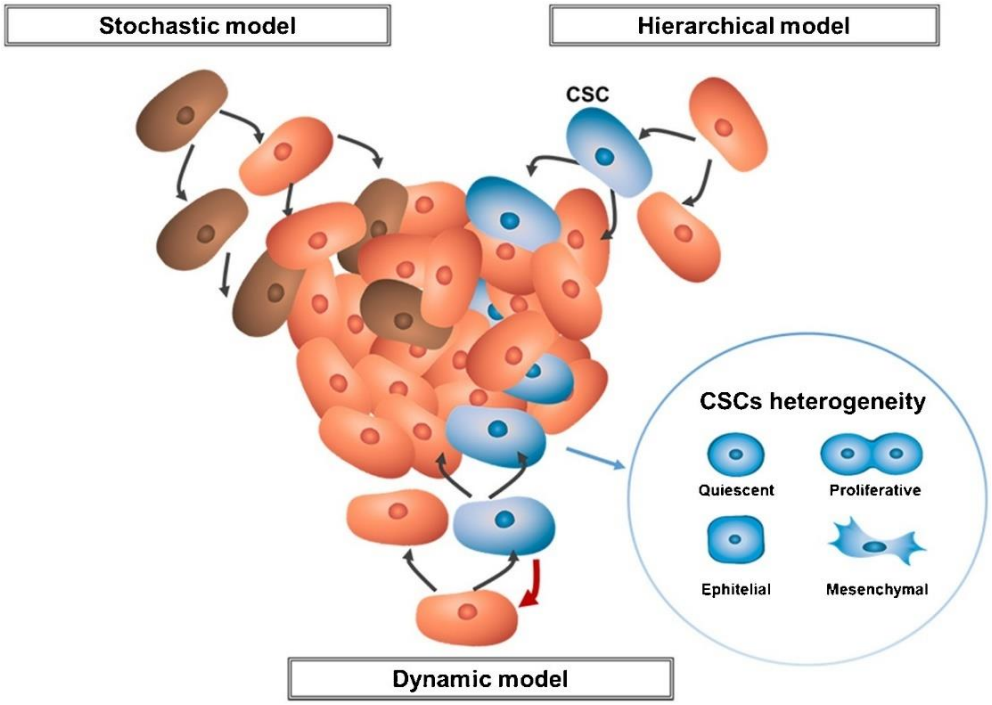


Figure 5. Three different models to define tumor origin and growth (source: reference 40)

As healthy tissues, tumors contain a cellular heterogeneity with cells in different stadiums of differentiation. CSC population possess the same properties as stem cells from healthy tissues –renewal and differentiation–, although in CSCs these processes are uncontrolled breaking down homeostatic equilibrium. CSCs have an important role in tumorigenesis, since a small number of these cells are capable of initiating a tumor. CSC population is not a static model; they have the capacity of multiple differentiation into any cell type resulting in a metastatic process. Also, these cells are in a quiescent state, it means, they have a slow cellular cycle, which may partly explain their role in therapy resistance (Figure 6).[41]

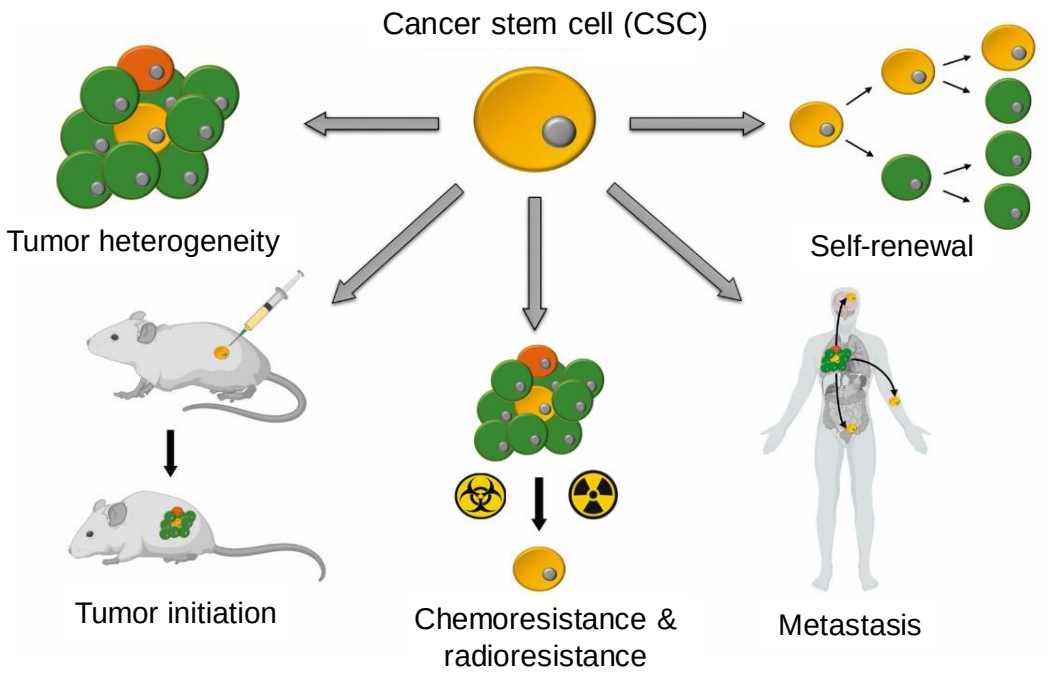


Figure 6. Properties of cancer stem cells (CSCs)

One of the most enlightening discoveries has been the identification of CSCs. This subpopulation shows specific surface markers that are not present in non-stem cells.[42] In the 1970s it was observed that stem cells existed into tumors and were able to form colonies *in vitro*.[43] This theory was provided by Dick in the 1990s when he identified and isolated CSCs from human acute myeloid leukemia (AML).[44] They sorted $CD34^{+}/CD38^{-}$ cells (representing 1% of total population), with similar phenotype to hematopoietic cells, and realized that these cells were the only ones capable to induce leukemia in a xenograft assay. Clark and his colleagues applied similar experiments to a solid breast cancer, isolating $CD44^{high}/CD24^{low}$ stem cells (representing 2% of total population).[45] CSCs were also identified in other solid tumors such as brain,[46] colon,[47, 48] pancreas,[49] and ovarian[50] cancers, and the list has grown, e.g. liver,[51] lung,[52] head and neck,[53] among others (Table 1). These experiments support the CSC model for carcinogenesis.

Table 1. Identified CSC markers in several types of cancers (source: reference 42)

Cancer type	**CSC markers**
AML	$CD34^{+}CD38^{-}$
brain	$CD133^{+}$, $CD44^{+}$
breast	$CD24^{-/low}CD44^{+}$, $ALDH^{bright}$, $CD133^{+}$, $CD221^{+}$
colon	$CD133^{+}$, $CD44^{+}$, $CD24^{+}$, $CD166^{+}$, $Lgr5^{+}$, $ALDH^{bright}$
head and neck	$CD133^{+}$, $CD44^{+}$, $ALDH^{bright}$, SP, $GRP78^{+}$, c-Met^{+}
liver	$CD133^{+}$, $CD90^{+}$, $EpCAM^{+}/CD44^{+}$, $CD13^{+}$, SP
lung	$CD44^{+}$, $CD133^{+}$, $CD117^{+}$, $CD87^{+}$, SP, $ALDH^{bright}$
ovarian	SP, $CD133^{+}$, $CD44^{+}$, $CD24^{+}$, $CD117^{+}$, $EpCAM^{+}$, $ALDH^{bright}$
pancreatic	$CD44^{+}/CD24^{+}/ESA^{+}$, $CD133^{+}$, c-Met^{+}, $ALDH^{bright}$
prostate	$CD44^{+}CD24^{-}$, $CD44^{+}/CD133^{+}/\alpha2\beta1^{high}$, $CD44^{+}/CD133^{+}/ABCG2^{+}/CD24^{-}$

AML is a type of cancer that originates in the myeloid line of hematopoietic stem cells. When hematopoiesis is normal, the myeloid precursor cells in the bone marrow maturate and are transformed in blood cells (erythrocytes, platelets, granulocytes, macrophages, etc.) (Figure 7). In contrast, in a patient with AML, there is a maturational stoppage of myeloid precursors, myeloblasts, in the bone marrow. AML starts in the bone marrow but can quickly pass to blood. The blast accumulation displaces normal hematopoietic tissue, which causes spinal insufficiency (leukopenia, anemia and thrombocytopenia), and infiltrates other extramedullar organs (liver, spleen, skin, nervous system, etc.).[54]

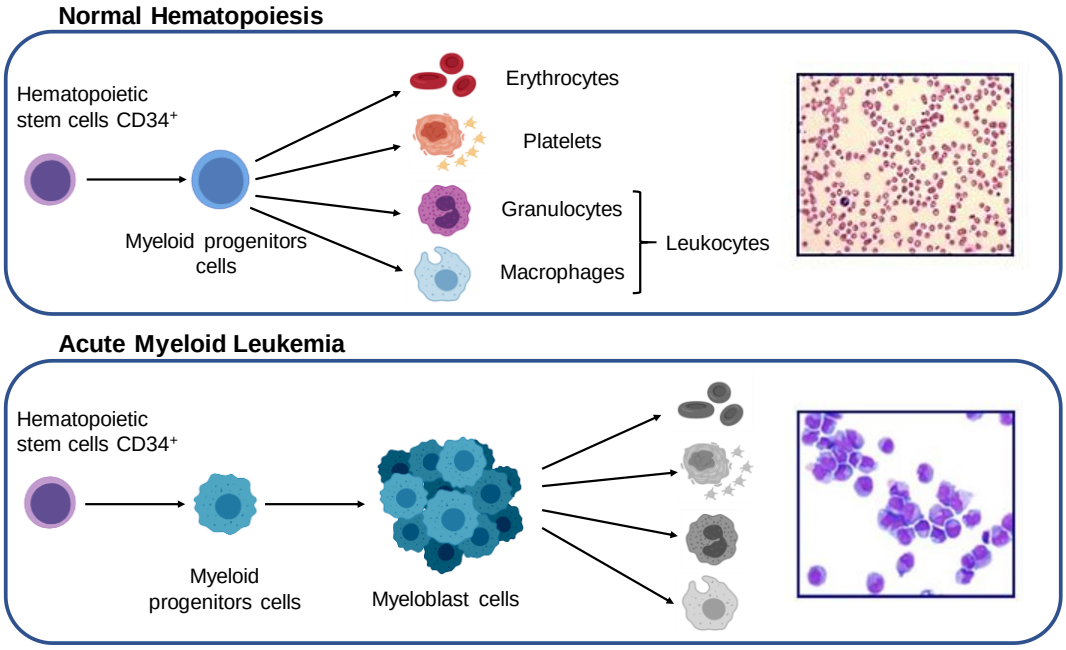


Figure 7. Myeloid hematopoiesis in healthy *vs* AML conditions

Current treatment(s) are still largely based on the combination of conventional chemotherapy and the appropriate use of stem cell transplantation, but in many cases these therapies are not enough, causing relapses.[55] Today, new drugs are being developed to treat AML, such as molecularly target agents against mutant driver proteins (e.g. midostaurin, quizartinib).[56]

In general, AML is fatal for the majority of patients, and recent studies show that patients younger than 55 to 60 years of age have about 40% 5-year survival, but those older than 60 years have an abysmal less than 5% to 10% 5-year survival. Consequently, we have seen rising AML mortality rates as the aging population continues to increase despite some improvements in the treatment of younger patients. Clearly, new and better therapies are urgently needed for AML treatment, specifically for older patients.

As CSCs are more resistant to treatments than non-stem cancer cells and are responsible for the development of the disease, a promising therapeutic strategy is to eliminate those CSCs or induce their differentiation process.[57-59] Hence, treatments targeting to remove the stemness will lead to a remaining population of non-stem cancer cells that should respond to conventional therapies to be eradicated (Figure 8).

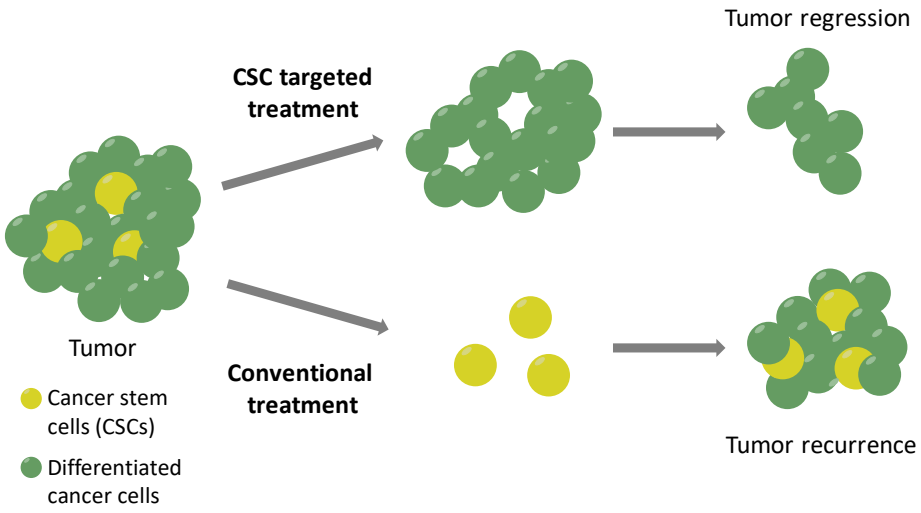


Figure 8. CSC targeted therapies for cancer treatment

1.3. Objectives

Taking into account the influence of the human microbiota in cancer, and the importance of targeting CSCs to find new therapies for the treatment of cancer and metastatic processes, we propose to develop new compounds inspired on human microbiota metabolites that are capable of acting on CSCs. In the present work, this objective will be approached by encompassing the following aims:

1. Synthesis of new small molecules based on microbiota metabolites
2. *In-vitro* CSC model: Cellular phenotypic screening based on cancer cell differentiation
3. *Ex-vivo* CSC model: Study of selected compound(s) in blood samples from AML patients

RESULTS AND DISCUSSION

2. RESULTS AND DISCUSSION

2.1. Synthetic approach of microbiota-inspired molecules

Despite the growing evidence that microbiota metabolites have beneficial effects on human health, challenges remain in the translation of the utility of these bioactive molecules into clinical therapeutics. Indeed, we probably only know a small fraction of the existing bioactive molecules in our microbiome. Chemists and chemical biologists have contributed to the isolation and characterization of approximately 115,000 small-molecule metabolites found in the human body, which are collected in the Human Metabolome Database[60] and the Virtual Metabolic Human.[61] Some representative examples, resulting from the bioconversion of dietary components, and their biological role in human health and disease are described in Figure 9. In particular, short-chain fatty acids –such as acetate, propionate, and butyrate–, vitamins –e.g. folic acid and riboflavin–, indoles, neurotransmitters –e.g. GABA, noradrenaline, and serotonin–, and phytoestrogens –e.g. urolithins and equol– play a major role in host energy storage, nutrition, immune functionality, central nervous system function, and cancer protection, respectively.[29] The metabolites of the microbiota therefore represent an interesting starting point for the design of new compounds for drug discovery.

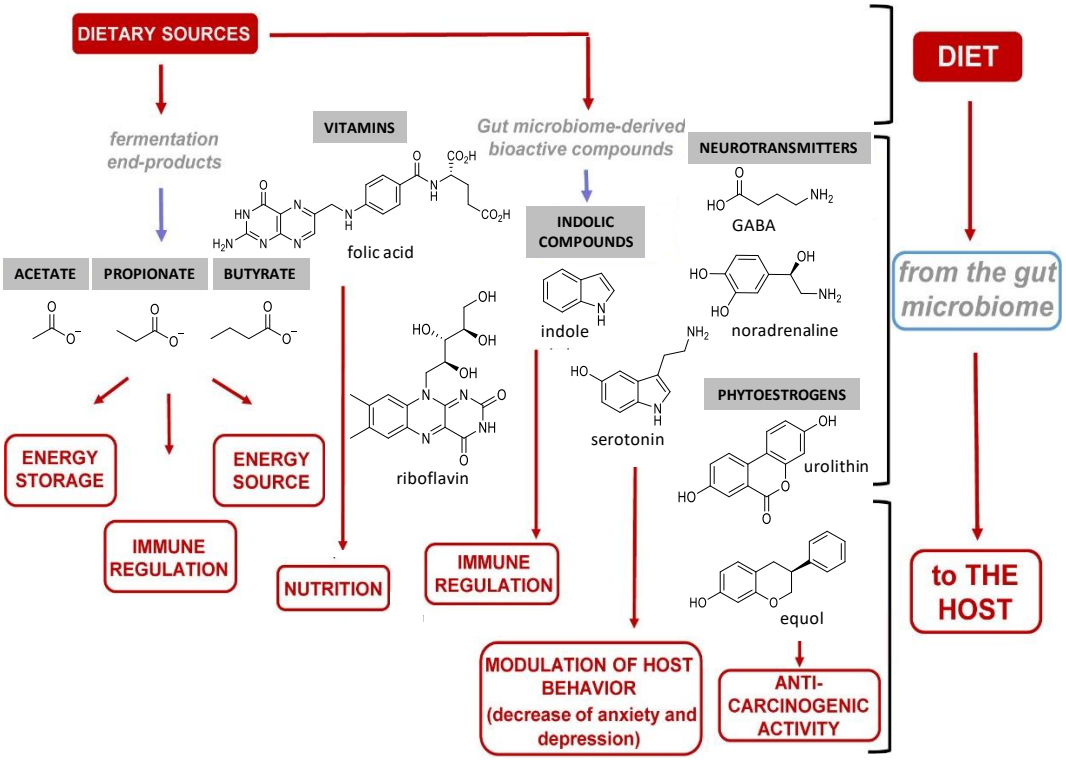


Figure 9. Representative human microbiota metabolites (source: reference 29)

Organocatalysis, the use of small organic molecules as catalysts, has a great impact in chemistry. Asymmetric organocatalytic domino reactions between two or more starting materials are versatile synthetic tools to access structurally diverse sets of complex scaffolds with high efficiency. Molecules can be assembled in a one-pot process, which involves the formation of a number of bonds in a single operation without isolating the intermediates or changing the reaction conditions. Therefore, these reactions are ideally suitable for the design and synthesis of diversity- and/or biology-oriented focused libraries, to obtain highly functionalized molecules in a straightforward and stereocontrolled manner.[62, 63]

Despite of the extensive development of organocatalysis, their application in the synthesis of active compounds in medicinal chemistry has not been widely exploited. In recent years, asymmetric organocatalytic reactions have been added to the drug discovery process as a valuable synthetic methodology for the preparation of building blocks and the production of diverse small-molecule libraries.[64, 65] Indeed, several groups have used organocatalytic reactions in total synthesis of bioactive natural products, some clinical drug candidates as neuroprotective, antiviral and antitumor agents, and they have also been

incorporated in new more efficient synthetic routes of drugs currently available in the market such as oseltamivir (Tamiflu®).[65, 66]

In this project, we propose to take advantage of asymmetric organocatalytic reactions as a key step to generate structurally diverse compounds based on microbiota metabolites, with high efficiency and in a stereocontrolled manner. Toward this end, we have analysed the structures of identified small-molecule metabolites[60] and the different types of organocatalytic reactions. Thus, privileged scaffolds (PrSc) present in the microbiota metabolites represented in Figure 10A were selected to design new compounds. The general structure of the new molecules consists of a PrSc-based central core to which other PrSc are linked, and a drug-like fragment to improve the pharmacokinetic properties (Figure 10B). In the designed compounds **1**-**20** (Figure 10C) the central core is a piperidinone or a piperidine, present in 3-amino-2-piperidone or pipecolic acid, respectively; a tetrahydrocarbazole, based on methyl 3-carbazolecarboxylate and related metabolites; a chromane, present in equol; a dihydropyrido[2,3-*b*]pyrazine, based on folic acid and related metabolites; or a 1,3,4,5-tetrasubstituted cyclohexene present in shikimic acid. In all cases a *p*-methoxyphenyl and an amino group are also attached to the central core, as these PrSc are present in a wide variety of metabolites. In some compounds, additional PrSc are introduced: an aliphatic chain, based on short-chain fatty acids; a pyridinecarboxamide, present in vitamin B3; an indole, present in tryptamine and related metabolites; or a thiazole ring, based on vitamin B1.

Regarding the synthesis of the designed compounds, the first step of the synthetic approach (Figure 10B) is an asymmetric organocatalytic reaction, specifically an aminocatalytic reaction, between an α,β-unsaturated aldehyde and a nitrocompound, using an enantiopure prolinol derivative ((*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine) as catalyst. The role of the amine catalyst is to promote the electrophile or nucleophile activation of the carbonyl substrate, via iminium or enamine intermediates, in addition to create a chiral environment to obtain enantiomerically enriched compounds. Considering that we are searching for new drug candidates, the products resulting from this reaction will be further derivatized: the nitro group will be reduced to amino, and the aldehyde transformed into a drug-like fragment, such as (cyclopropylmethyl)amine.

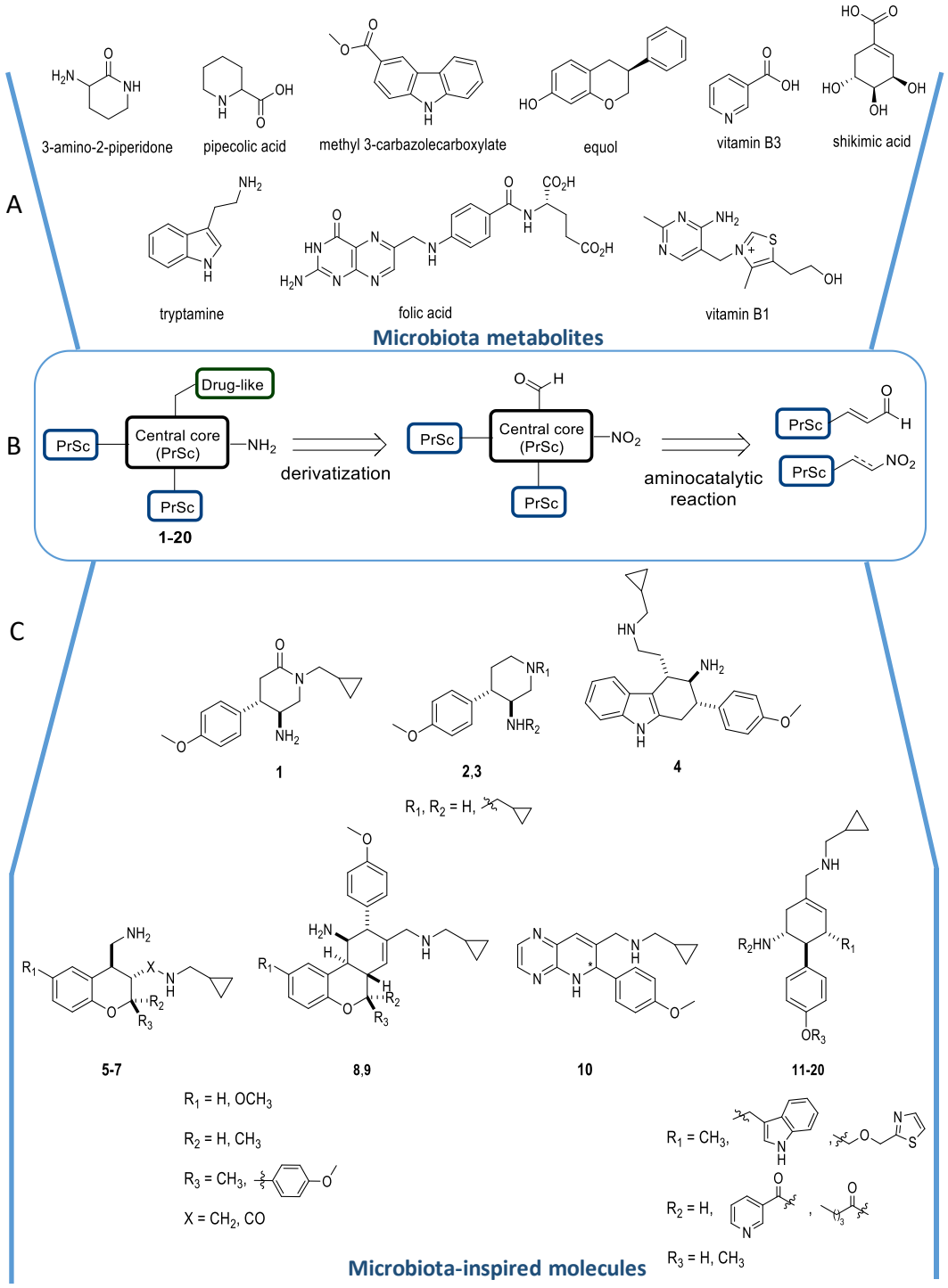


Figure 10. Microbiota metabolites (A) selected for the design of microbiota-inspired molecules **1**-**20** (C) and synthetic approach via amino organocatalytic reactions (B)

Throughout this section, synthesized compounds have been drawn using unwedged bold and dashed lines to represent the relative configuration of the chiral centers, and the corresponding *R* and *S* designations have been included next to the number assigned to each compound to indicate the absolute configuration of chiral centers.

2.2. Synthesis of piperidine scaffold. Final compounds 1-3

According to a synthetic methodology described to obtain 2-piperidinone and piperidine derivatives with good diastereoselectivity and excellent enantioselectivity,[67] we proposed the obtention of final compounds **1**-**3** by an asymmetric organocatalytic Michael addition of Boc-protected 2-amino-1-nitroethane **21** to *p*-methoxycinnamaldehyde acting as *α*,*β*-unsaturated aldehyde, followed by cyclization (Figure 11).

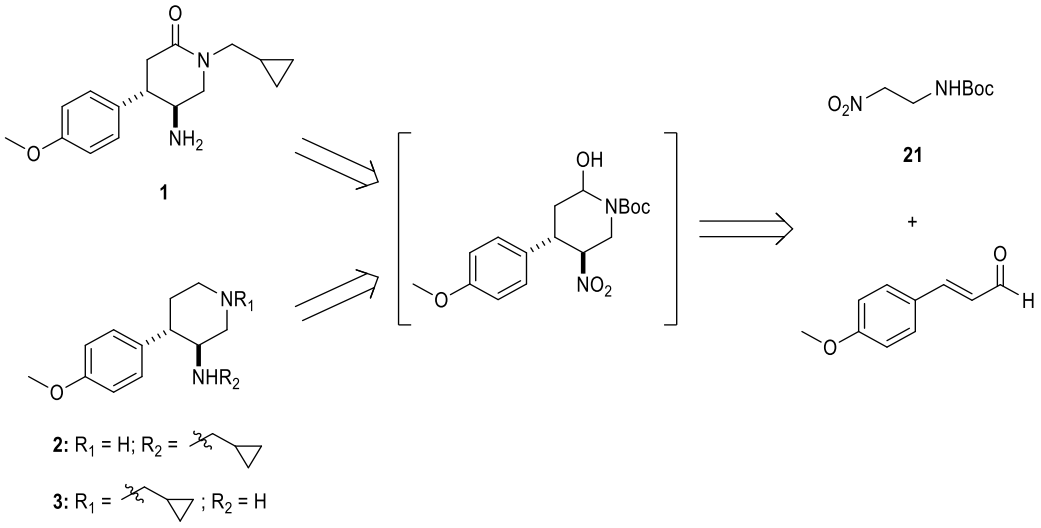
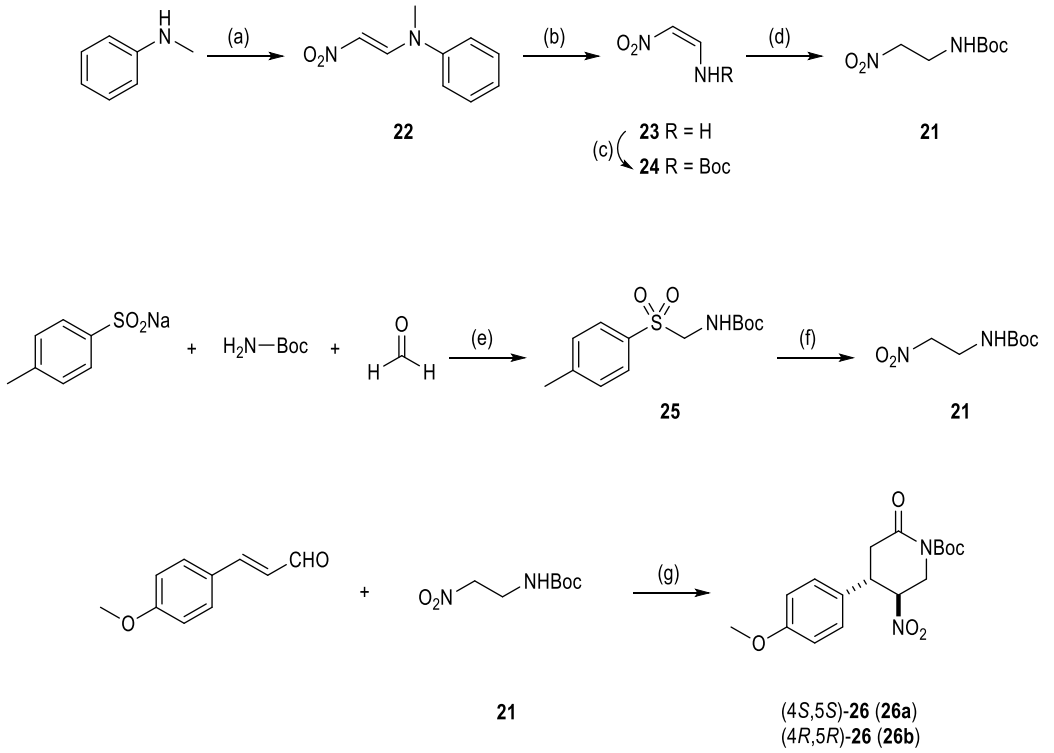


Figure 11. Synthetic approach for compounds **1**-**3**, containing a piperidine scaffold

For the preparation of starting compound **21**, a methodology previously reported was followed (Scheme 1).[67, 68] Thus, the condensation of *N*-methylaniline, nitromethane and triethyl orthoformate gave 2-nitroenamine **22**, whose treatment with an ammonia solution in dioxane yielded (*Z*)-2-nitroethenamine **23** by transamination. Then, amine **23** was protected with di-*tert*-butyl dicarbonate to obtain *N*-Boc-2-amino-1-nitroethene **24**, which was transformed into the desired compound **21** by reduction of the alkene using sodium borohydride. Since the global yield achieved in this route was very low (10%), we decided to set up an

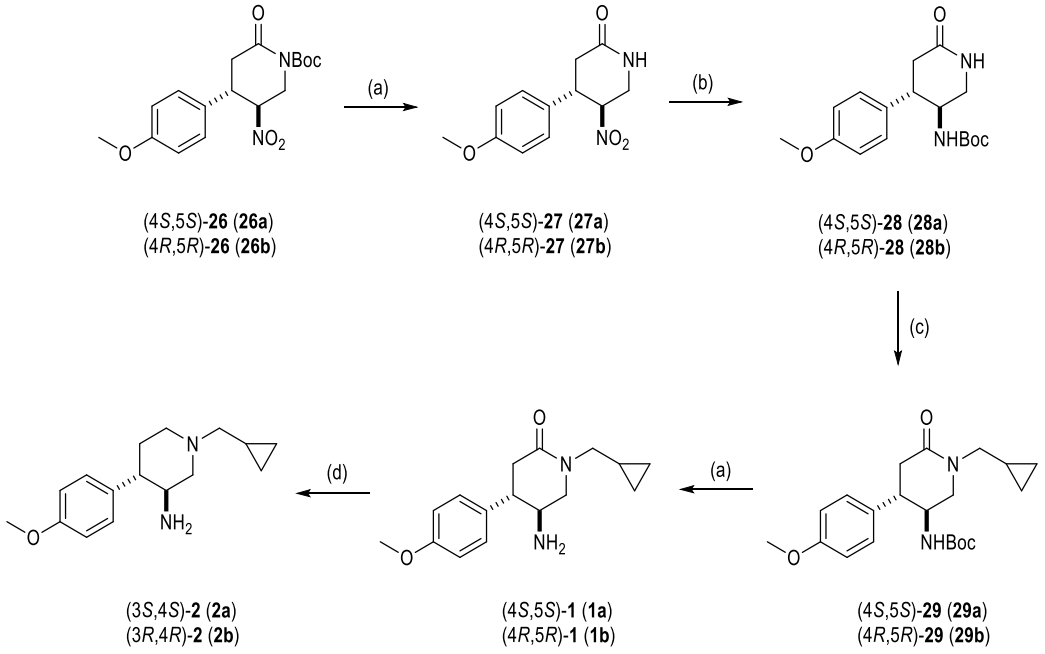
alternative pathway to prepare starting compound **21**. The first step consisted in the synthesis of sulfone **25** from *tert*-butyl carbamate, *p*-toluene sulfinic acid (sodium salt) and paraformaldehyde. Next, the microwave (MW)-assisted aza-Henry reaction of **25** with nitromethane in the presence of potassium carbonate led to the desired compound **21** (Scheme 1). This new synthetic route allowed us to prepare the protected 2-amino-1-nitroethane **21** in only two steps and with a better overall yield (50%).

The organocatalytic Michael addition of **21** to *p*-methoxycinnamaldehyde using the enantiopure *S* or *R* form of 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine as the catalyst and benzoic acid as the additive gave the corresponding cyclic hemiaminal, which was *in situ* oxidized with pyridinium dichromate (PDC) to provide enantiomers **26a** or **26b**, respectively, with high diastereoselectivity (*trans*/*cis* ratio = 9:1, determined by ^{1}H-nuclear magnetic resonance (NMR) analysis of the crude) (Scheme 1). The relative *trans* configuration for nitro and *p*-methoxyphenyl groups in compound **26a** was assigned using ^{1}H-NMR nuclear Overhauser effect (NOE) experiments. Thus, NOE was observed for $CHNO_2$ proton and aromatic protons. The absolute configuration of the enantiomers was assigned according to the stereochemical outcome of the reported asymmetric organocatalytic reaction. [67]



Scheme 1. Reagents and conditions: (a) CH_3NO_2, $CH(OEt)_3$, cat. *p*-TsOH, reflux, 7 h, 45%; (b) NH_3 (0.5 M in 1,4-dioxane), 5 ºC, on, 43%; (c) Boc_2O, cat. DMAP, DCM, rt, 15 min, quantitative; (d) $NaBH_4$, MeOH, 0 ºC to rt, 2 h, 52%; (e) HCOOH, MeOH/H_2O, reflux (1.5 h) to rt, on, 60%; (f) CH_3NO_2, K_2CO_3, THF, MW, 120 ºC, 30 min, 50%; (g) i. 10 mol% chiral cat., 20 mol% benzoic acid, DCM, 0 ºC to rt, 5 h; ii. PDC, rt, on, 45-58%.

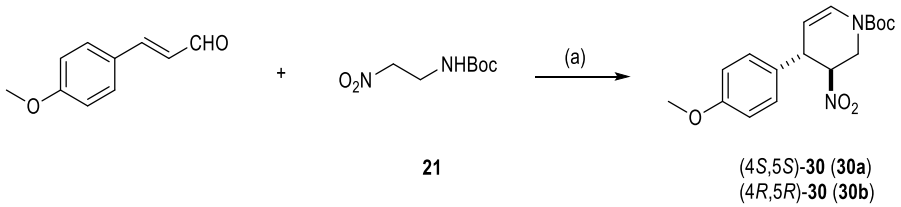
Intermediate **26a** was treated with trifluoroacetic acid (TFA) to remove the Boc-protecting group, affording lactam **27a** (diastereomeric ratio, dr = 85:15) (Scheme 2). Reduction of the nitro group and Boc-protection of the resulting amine were carried out in a one-pot reaction, using *in situ* generated nickel boride as reducing agent and di-*tert*-butyl dicarbonate, to give intermediate **28a** (dr = 85:15). Next, the amide group was alkylated with (bromomethyl)cyclopropane using sodium hydride as base to afford compound **29a** (dr = 85:15). The major *trans* diastereoisomer was isolated in pure form by flash chromatography and subsequently treated with TFA to remove the Boc-protecting group, to yield final compound **1a**. Finally, the carbonyl group of **1a** was reduced with lithium aluminum hydride to obtain target compound **2a** (Scheme 2). Starting from enantiomer **26b**, final compounds **1b** and **2b** were synthesized according to Scheme 2. Both enantiomers of **1** and **2** were obtained with high enantiomeric excess, *ee* (96%).



Scheme 2. Reagents and conditions: (a) TFA, DCM, rt, 45 min-4 h, 73-93%; (b) i. $NiCl_2 \cdot 6H_2O$, $NaBH_4$, MeOH, 0 ºC, 1 h; ii. Boc_2O, rt, on, quantitative; (c) i. NaH, DMF, 0 ºC, 1 h; ii. (bromomethyl)cyclopropane, NaI, rt, on, 36-42%; (d) $LiAlH_4$, THF, rt, on, 77-85%.

The same asymmetric organocatalytic Michael addition of **21** to *p*-methoxycinnamaldehyde was applied to the synthesis of final compound **3**. In this case, the aminocatalytic reaction was followed by *in situ* dehydration of the resulting cyclic hemiaminal with TFA to provide tetrahydropyridine **30a** or **30b** (Scheme 3), with high diastereoselectivity (dr = 8:2, determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis of the crude).

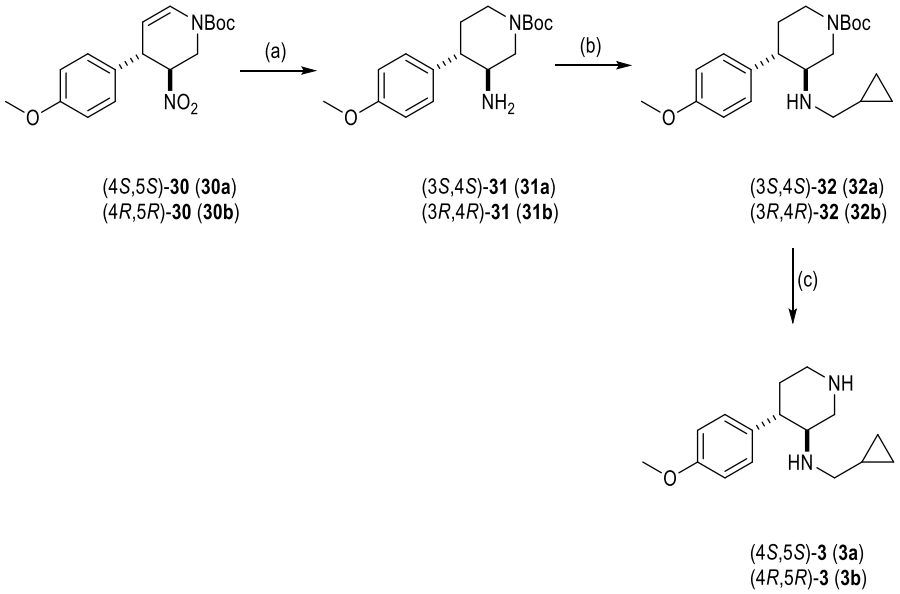
Reduction of both the nitro group and the double bond of intermediate **30a** was carried out using nickel boride, yielding aminopiperidine **31a**, which was isolated as a single pure diastereoisomer after flash chromatography (Scheme 4). Reductive amination of cyclopropranecarbaldehyde with **31a** in the presence of sodium borohydride provided intermediate **32a** which was deprotected to afford final compound **3a**. Enantiomer **3b** was obtained using the same derivatization shown in Scheme 4 starting from intermediate **30b**. Final compounds **3a** and **3b** were obtained with high *ee* (96%).



21

(4*S*,5*S*)-**30** (**30a**)
(4*R*,5*R*)-**30** (**30b**)

Scheme 3. Reagents and conditions: (a) i. 10 mol% chiral cat., 20 mol% benzoic acid, DCM, 0 ºC to rt, 5 h; ii. TFA, rt, 2 h, 84-87%.



(4*S*,5*S*)-**30** (**30a**)
(4*R*,5*R*)-**30** (**30b**)

(3*S*,4*S*)-**31** (**31a**)
(3*R*,4*R*)-**31** (**31b**)

(3*S*,4*S*)-**32** (**32a**)
(3*R*,4*R*)-**32** (**32b**)

(4*S*,5*S*)-**3** (**3a**)
(4*R*,5*R*)-**3** (**3b**)

Scheme 4. Reagents and conditions: (a) $NiCl_2 \cdot 6H_2O$, $NaBH_4$, MeOH, 0 ºC to rt, on, 53-60%; (b) i. cyclopropranecarbaldehyde, MeOH, rt, 4 h; ii. $NaBH_4$, rt, 2 h, 63-64%; (c) TFA, DCM, rt, 3 h, 80-85%.

2.3. Synthesis of tetrahydrocarbazole scaffold. Final compound 4

The tetrahydrocarbazole scaffold present in target compound **4** was constructed using an asymmetric organocatalytic Diels-Alder reaction between β-indolyl α,β-unsaturated aldehyde **35** and a nitroalkene as dienophile (Figure 12).[69]

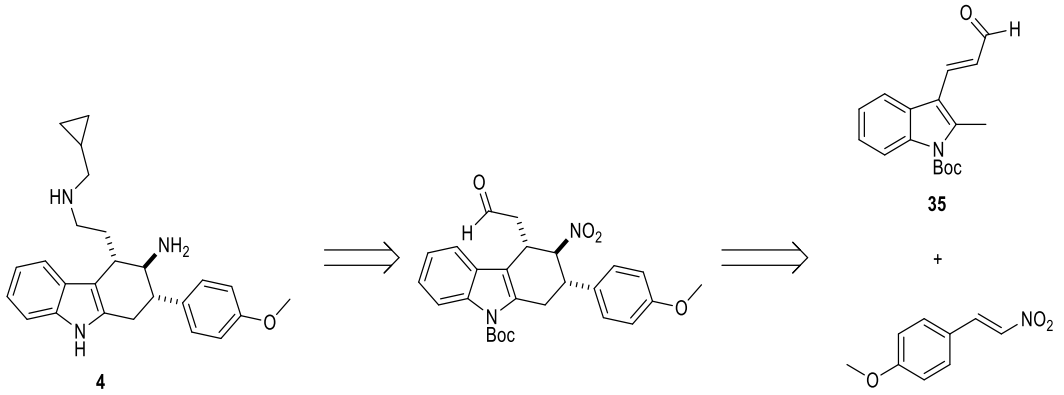
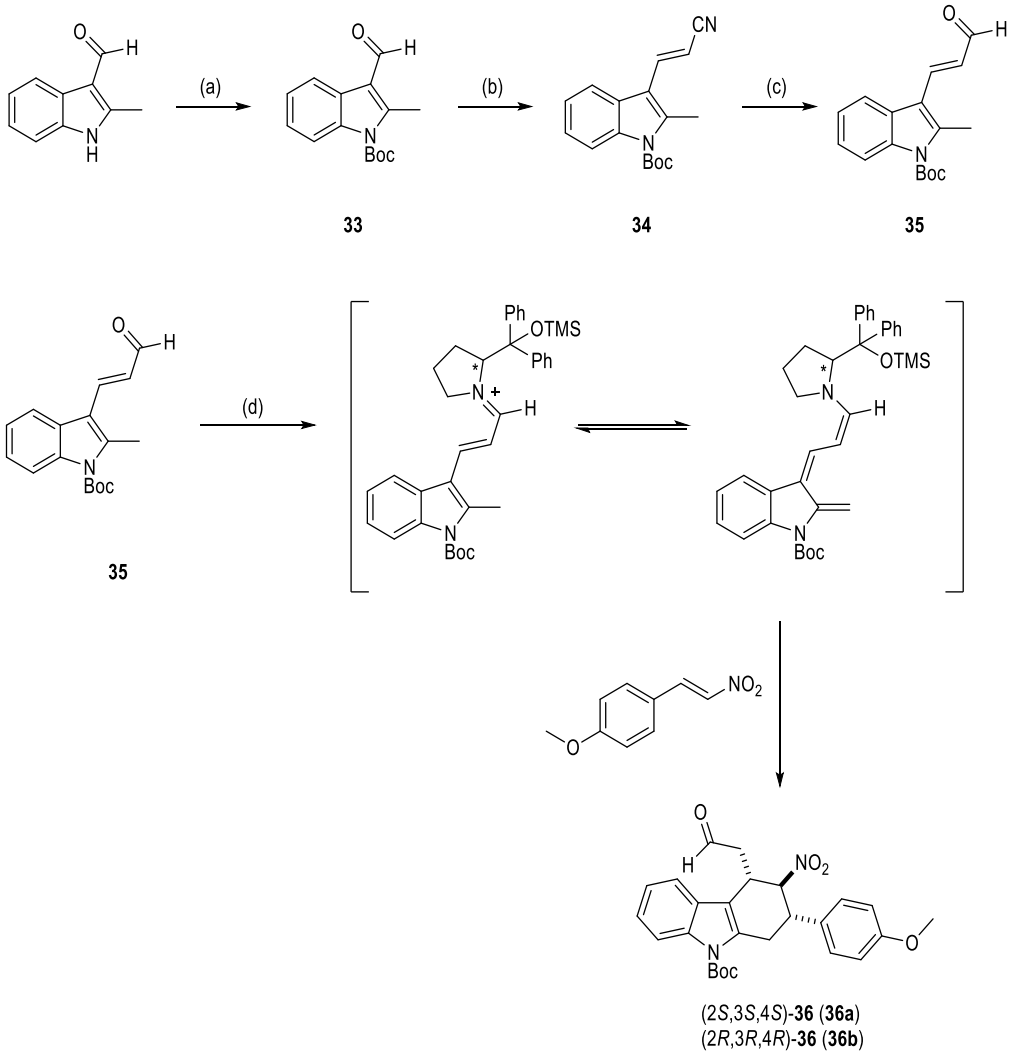


Figure 12. Synthetic approach for compound **4**, containing a tetrahydrocarbazole scaffold

Aldehyde **35** was synthesized from 2-methyl-1*H*-indole-3-carbaldehyde following the described procedure.[69] Thus, Boc-protection, subsequent Horner-Wadsworth-Emmons reaction and final nitrile reduction with diisobutylaluminum hydride (DIBALH) allowed to obtain the diene precursor **35** (Scheme 5).

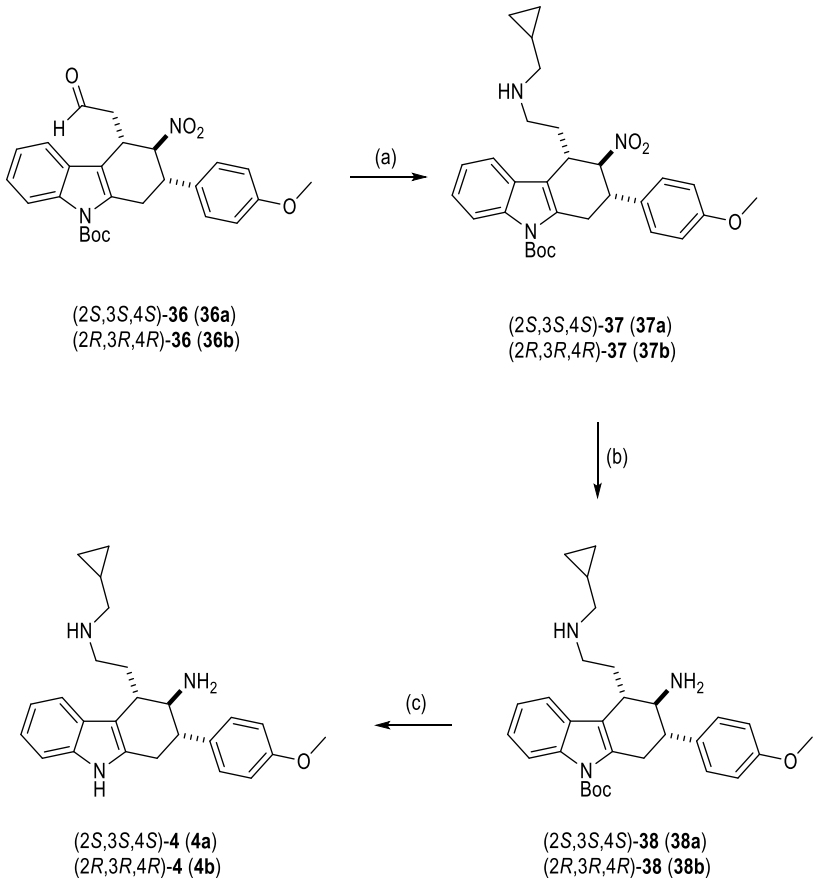
The organocatalytic Diels-Alder reaction of **35** and *trans*-*p*-methoxy-*β*-nitrostyrene was carried out using the enantiopure *S* or *R* form of 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine as the catalyst and benzoic acid as additive. In this reaction, the first step is the generation *in situ* of the corresponding *ortho*-quinodimethane from **35**, by reaction with the catalyst and subsequent tautomerization. This intermediate selectively directs the [4+2] cycloaddition with the dienophile toward a highly diastereo- and enantioselective pathway to yield tetrahydrocarbazole enantiomer **36a** or **36b** (dr = 98:2, determined by ^{1}H-NMR analysis of the reaction crude) (Scheme 5). The major diastereoisomer was isolated in pure form by flash chromatography and its relative configuration was assigned by NOE experiments. Thus, the relative *trans* configuration for nitro and *p*-methoxyphenyl groups was confirmed due to NOE between the aromatic protons and $CHNO_2$ proton; the *trans* configuration for nitro and formylmethyl groups was determined by means of NOE between methylene protons and $CHNO_2$ proton. The absolute configuration of the enantiomers was assigned based on the results of the reported asymmetric organocatalytic reaction.[69]



Scheme 5. Reagents and conditions: (a) Boc_2O, DMAP, ACN, rt, 4 h, 90%; (b) diethyl cyanomethylphosphonate, BuLi, THF, 0 ºC, 4 h, 85%; (c) DIBALH, toluene, -78 ºC, 4 h, 89%; (d) 20 mol% chiral cat., 20 mol% benzoic acid, toluene, 70 ºC, 40 h, 45-53%.

Compound **36a** was further functionalized via reductive amination to introduce a (cyclopropylmethyl)amino moiety, followed by reduction of the nitro group by treatment with zinc in a mixture of acetic acid/methanol, and subsequent Boc deprotection with hydrochloric acid to afford final compound **4a** (Scheme 6).

Intermediate **36b** was likewise derivatized according to Scheme 6 to give enantiomer **4b**. Final compounds **4a** and **4b** were obtained with high *ee* (93%).



Scheme 6. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH/DCM, rt, 3 h; ii. $NaBH_4$, rt, 2 h, 83-86%; (b) Zn, AcOH/MeOH, 2 h, quantitative; (c) HCl, MeOH, rt, 4 h, 40-50%.

2.4. Synthesis of chromane and tetrahydrobenzo[*c*]chromene scaffolds. Final compounds 5-9

In order to obtain compounds **5**-**9**, we proposed to apply an asymmetric organocatalytic domino reaction between an *o*-nitrovinilphenol and an α,β-unsaturated aldehyde (Figure 13). This reaction has been reported to provide chromane and/or tetrahydrobenzo[*c*]chromene scaffolds with excellent diastereo- and enantioselectivity through a domino *oxa*-Michael-Michael reaction and a quadruple cascade *oxa*-Michael-Michael-Michael-aldol condensation reaction, respectively (Figure 14).[70]

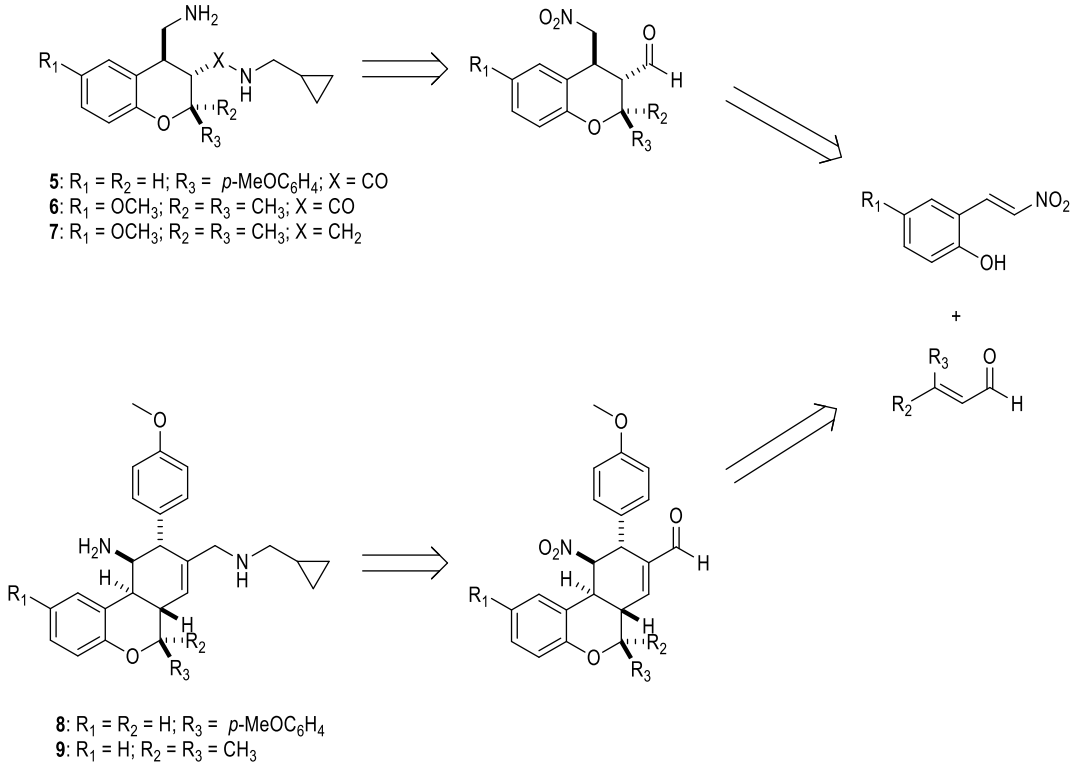


Figure 13. Synthetic approach for target compounds **5**-**9**, containing chromane or tetrahydrobenzo[*c*]chromene scaffolds

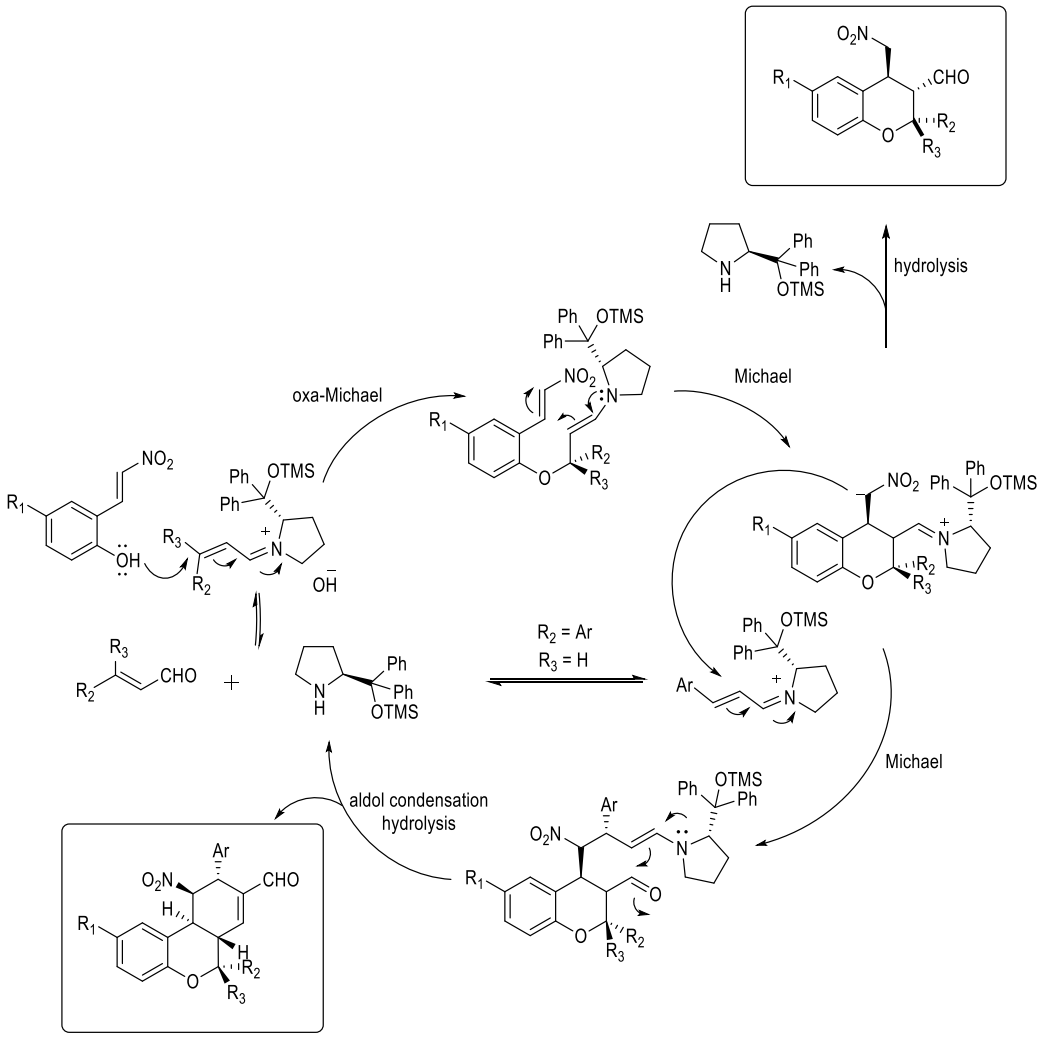


Figure 14. Proposed mechanism reported for the cascade reaction to provide chromane and tetrahydrobenzo[*c*]chromene scaffolds

For the synthesis of those compounds **5**-**9** where R_2 = H and R_3 = *p*-$MeOC_6H_4$, *p*-methoxycinnamaldehyde was used as α,β-unsaturated aldehyde, and we adapted the reported conditions that establish that both chromane and tetrahydrobenzo[*c*]chromene scaffolds can be formed in a 1:1 ratio from *o*-nitrovinylphenol and cinnamaldehyde.[70] Thus, the reaction between *o*-nitrovinylphenol and three equivalents of *p*-methoxycinnamaldehyde was performed in anhydrous toluene at room temperature (rt), employing the *S* form of 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine as catalyst and *p*-nitrobenzoic acid (PNBA) as additive. However, the expected scaffolds **39a** and **40a** were not

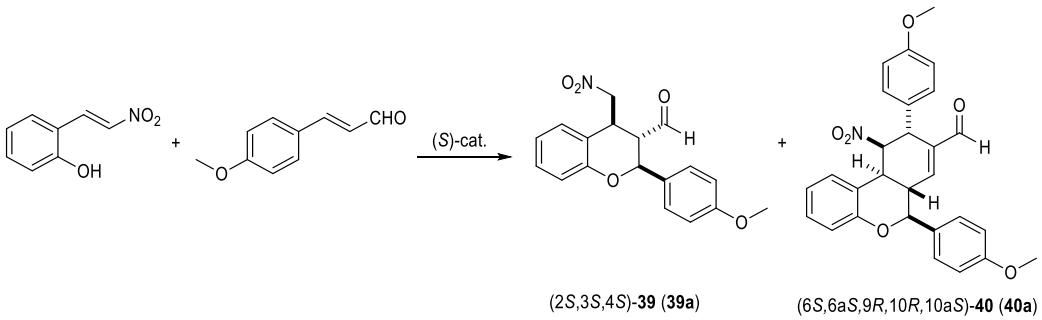
obtained, but starting material was recovered after 30 h of reaction (Table 2, entry 1).

We next explored modifications of the reaction conditions described to obtain both scaffolds. The equivalents of the aldehyde, the amount of the catalyst, the additive, the solvent and/or the time of the reaction were modified. In an initial modification, chloroform was used as solvent in the absence of additive and a 2:8 mixture of **39a** and **40a** was obtained (Table 2, entry 2). Then, reduction of the equivalents of the aldehyde and addition of acetic acid afforded **39a** as the major product after 5 h (Table 2, entries 3, 5 and 6) or a 1:1 mixture of **39a** and **40a** when kept overnight (Table 2, entry 4). In all cases, the dr for **39a** was 8:2 (determined by ^{1}H-NMR analysis of the reaction crude), whereas **40a** was obtained as a single diastereoisomer.

Therefore, the following conditions were established as the best procedure that allows direct access to both intermediates **39a** and **40a** in a 1:1 ratio: the reaction between *o*-nitrovinylphenol and 1.2 equivalents of *p*-methoxycinnamaldehyde in anhydrous chloroform at rt, employing 20 mol% of the *S* form of the chiral catalyst and acetic acid as an additive (Table 2, entry 4).

All the optimization reactions were carried out on a 0.3-mmol scale (50 mg) of *o*-nitrovinylphenol, and in order to obtain more quantity that allows the derivatization of intermediates **39a** and **40a**, we tried to scale up the reaction (Table 2, entries 7 and 8). In the reaction performed on a 1.2-mmol scale (200 mg) of *o*-nitrovinylphenol, starting material was recovered after 50 h of reaction, whereas the reaction conducted on a 0.6-mmol scale (100 mg) gave an equimolar mixture of both **39a** and **40a**. However, in the latter reaction, the diastereoselectivity observed for **39a** decreased (dr = 6:4, determined by ^{1}H-NMR analysis), with compound **40a** remaining as a single diastereoisomer.

Table 2. Experimental conditions of reaction to obtain compounds **39a** and **40a**

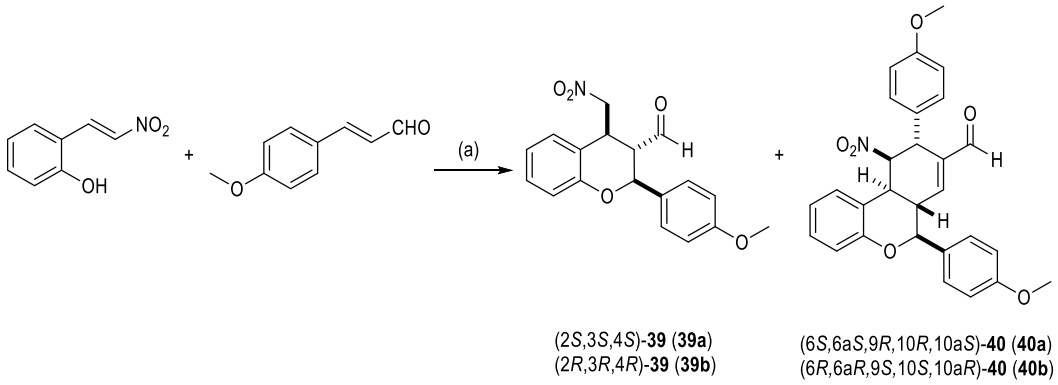


(2*S*,3*S*,4*S*)-**39** (**39a**) (6*S*,6a*S*,9*R*,10*R*,10a*S*)-**40** (**40a**)

Entry	Aldehyde (equiv)	Scale[a] (mmol)	Catalyst (mol %)	Additive	Solvent	t (h)	Product (39a/40a/SM)[b]
1	3	0.3	20%	PNBA[c]	Toluene	30	-/-/1
2	3	0.3	10%	-	$CHCl_3$	on[d]	2/8/-
3	1.2	0.3	20%	AcOH	$CHCl_3$	5	5/3/2
4	1.2	0.3	20%	AcOH	$CHCl_3$	on	2/2/1
5	1	0.3	20%	AcOH	$CHCl_3$	5	4/3/3
6	1	0.3	10%	AcOH	$CHCl_3$	5	2/1/2
7	1.2	1.2	20%	AcOH	$CHCl_3$	50	-/-/1
8	1.2	0.6	20%	AcOH	$CHCl_3$	on	2/2/1

[a]Scale referenced to mmol of *o*-nitrovinylphenol; [b]Determined in the reaction mixture by ^{1}H-NMR prior to work-up; SM (starting material): *o*-nitrovinylphenol; [c]*p*-nitrobenzoic acid; [d]on, overnight.

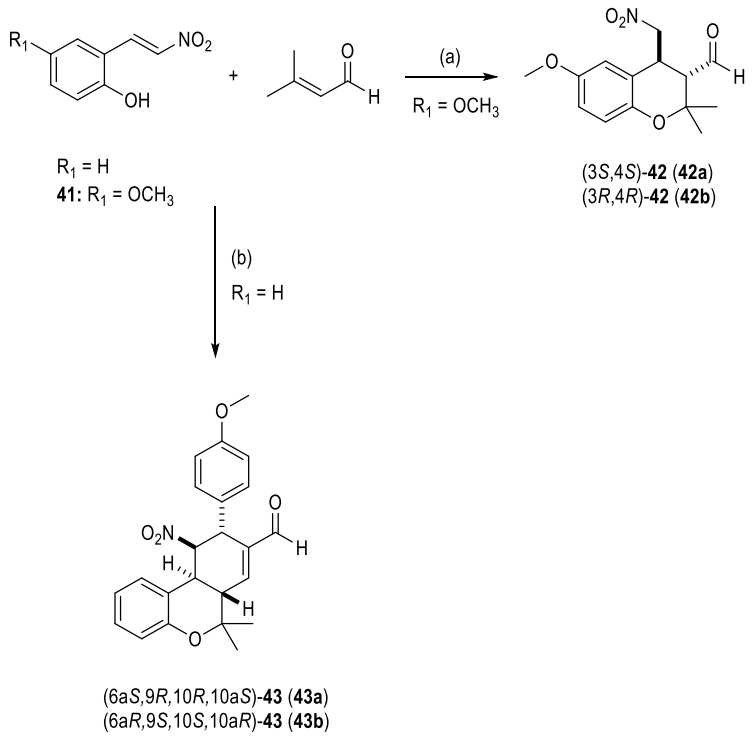
In view of these results, all the subsequent reactions to prepare **39** and **40** were conducted on a 0.3-mmole scale (50 mg) of *o*-nitrovinylphenol, using the appropriate enantiopure form of the prolinol derivative catalyst (Scheme 7). The separation of both intermediates **39** and **40** was carried out by flash chromatography, in which the major diastereoisomer of **39** was isolated in a pure form. The relative stereochemistry in compound **39a** was assigned using NOEs that were observed between the aldehyde proton and the corresponding β-protons, confirming the *trans* configuration for the aldehyde group and the other substituents in the chromane. In compound **40a**, NOEs between $CHNO_2$ proton, corresponding *p*-methoxyphenyl protons, and bridgehead proton, as well as between the other bridgehead proton and corresponding *p*-methoxyphenyl protons, determined their relative configurations. The absolute configuration of the enantiomers was assigned according to the stereochemical outcome of the reported asymmetric organocatalytic reaction. [70]



Scheme 7. Reagents and conditions: 20 mol% chiral cat., 20 mol% AcOH, $CHCl_3$, rt, on, 22-31% for **39**, 21-26% for **40**.

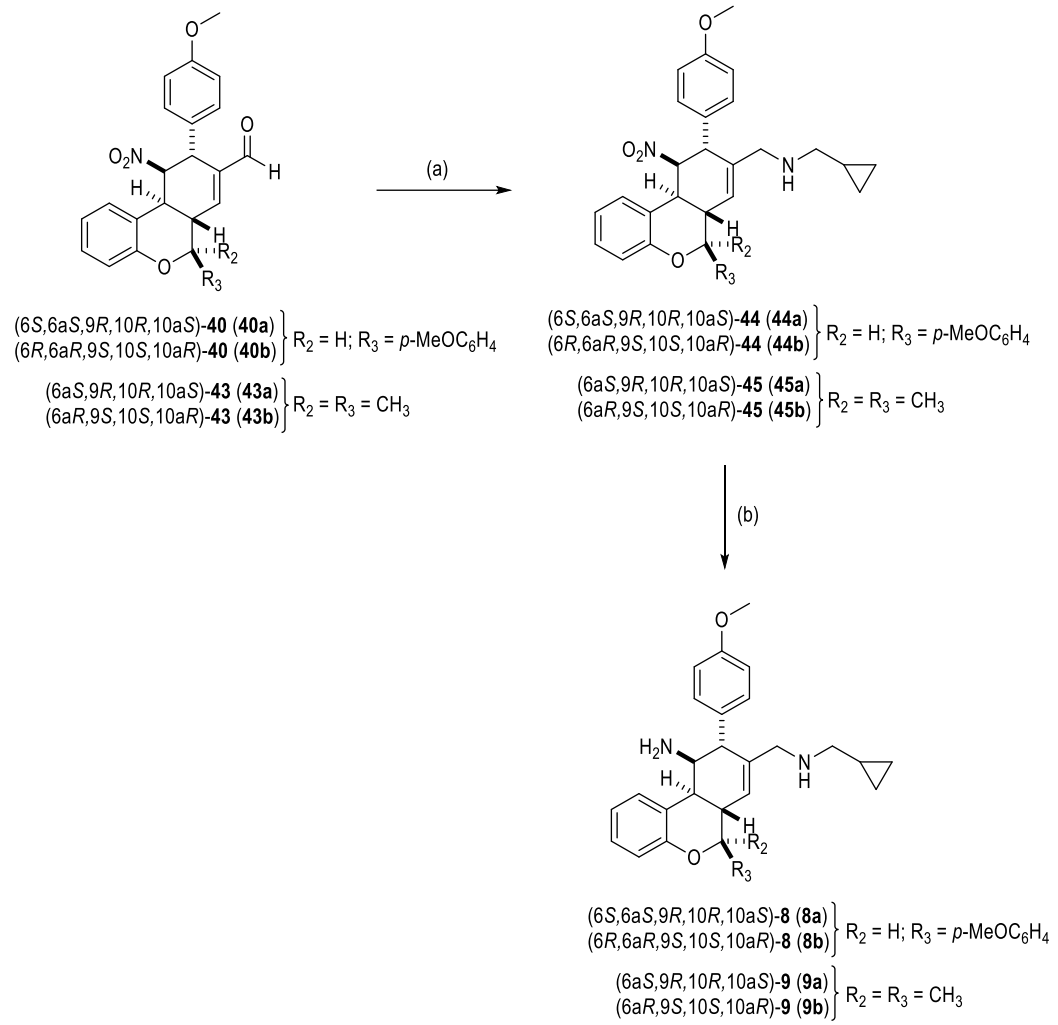
In the case of target compounds **5-9** with $R_2 = R_3 = CH_3$, the domino reaction between *trans*-2-hydroxy-5-methoxy-*β*-nitrostyrene (**41**) and 3,3-dimethylacrolein was carried out using the *S* or *R* form of the prolinol catalyst, affording only the *oxa*-Michael-Michael adduct **42a** or **42b** (dr = 8:2), respectively. In this case, the tetrahydrobenzo[*c*]chromene derivative was not formed, probably due to the steric hindrance of the *gem*-dimethyl groups, which may prevent the Michael addition to a third molecule of 3,3-dimethylacrolein. The relative *trans* stereochemistry for the major diastereoisomer of **42a** was confirmed by NOE experiments. Compound **41** was previously synthesized by Henry reaction of 2-hydroxy-5-methoxybenzaldehyde and nitromethane in the presence of ammonium acetate with good yield (60%).

Chromane derivative resulting from the *oxa*-Michael-Michael reaction between *trans*-2-hydroxy-*β*-nitrostyrene and 3,3-dimethylacrolein was not isolated, and *in-situ* addition of *p*-methoxycinnamaldehyde as a Michael acceptor induced a subsequent Michael-aldol condensation to give tetrahydrobenzo[*c*]chromene enantiomers **43a** or **43b** as a single diastereoisomer (Scheme 8).



Scheme 8. Reagents and conditions: (a) 20 mol% chiral cat., 20 mol% AcOH, $CHCl_3$, rt, 1 h, 52-61%; (b) *p*-methoxycinnamaldehyde, 20 mol% chiral cat., 20 mol% AcOH, $CHCl_3$, rt, 24 h, 43-48%.

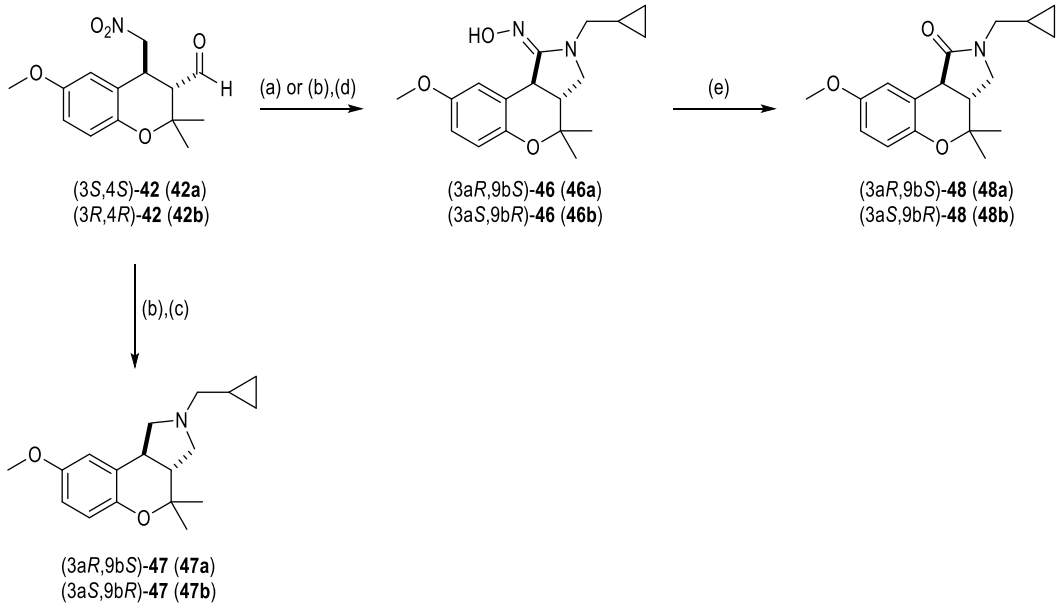
Next, according to the synthetic approach depicted in Figure 13, the derivatization of intermediates **39**, **40**, **42**, and **43** to obtain final compounds **5**-**9** was undertaken. The one-pot reductive amination of each enantiomer of tetrahydrobenzo[*c*]chromenes **40** and **43** by reaction with an excess of (cyclopropylmethyl)amine and *in situ* treatment with sodium borohydride provided the corresponding enantiomers of **44** and **45**, respectively (Scheme 9). Then, the reduction of nitro group by treatment with zinc in a mixture of acetic acid/methanol afforded final compounds **8** and **9**, respectively. Both enantiomers of **8** and **9** were obtained with high *ee* (96%), and the relative configuration of **8a** and **9a** was also assigned using NOE experiments.



Scheme 9. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH/DCM, rt, 3 h; ii. $NaBH_4$, rt, 2 h, 88%-quantitative (b) Zn, AcOH/MeOH, rt, 2 h, 41-93%.

Regarding the derivatization of the chromane intermediates **39** and **42**, the one-pot reductive amination of **42a** with (cyclopropylmethyl)amine did not afford the expected amine. In this case, unexpected oxime **46a** was obtained in low yield (Scheme 10, conditions (a)). The alternative reductive amination in two steps was also unsuccessful and cyclic amine **47a** was formed (Scheme 10, conditions (b)(c)). Then, we investigated a different protocol for the reduction of the corresponding imine, based on the use of trichlorosilane combined with *N*,*N*-dimethylformamide (DMF) as a soft Lewis base, since this methodology has been reported for the reduction of imines in the presence of a nitro group.[71] However, compound **46a** was obtained again in better yield (Scheme 10, conditions (b)(d)), and it was hydrolyzed to **48a**. Unexpected compounds **46a**-**48a** represent new

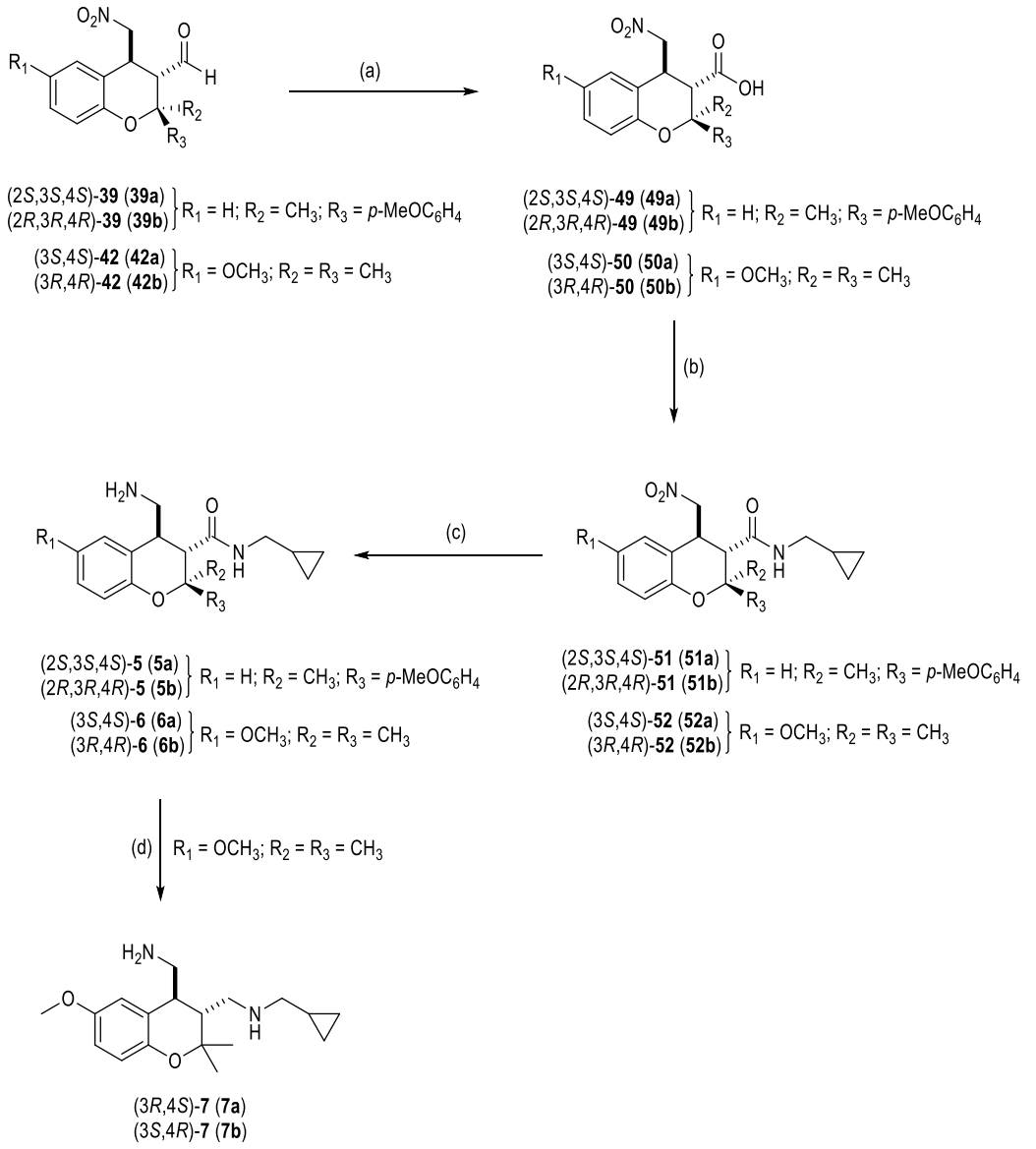
scaffolds that were considered as final compounds to be tested in the phenotypic assays. Thus, enantiomers **46b**-**48b** were synthesized using the same synthetic pathway, starting from intermediate **42b**. Both enantiomers of **46**-**48** were obtained, in all cases, with *ee* higher than 94%.



Scheme 10. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH, rt, 3 h; ii. $NaBH_4$, rt, 2 h, 17%; (b) (cyclopropylmethyl)amine, DCM, rt, 3 h, quantitative; (c) $NaBH_4$, MeOH, rt, 3 h, 30-33%; (d) $HSiCl_3$, DCM, DMF, rt, on, 80-89%; (e) $NaNO_2$, AcOH, MeOH/H_2O, rt, on, 30-40%.

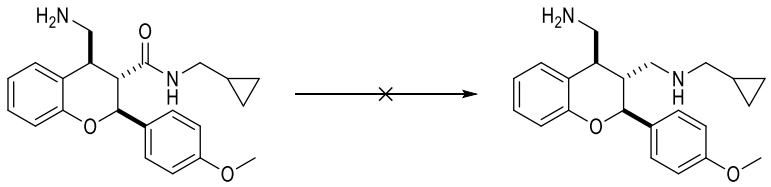
Hence, the derivatization of chromane intermediates **39a** and **42a** to final compounds **5a**-**7a** was performed following an alternative synthetic route as described in Scheme 11. Aldehydes were oxidized to the corresponding carboxylic acids **49a** and **50a** through Pinnick reaction, using sodium chlorite and a saturated solution of monopotassium phosphate as oxidizing system. (Cyclopropylmethyl)amine moiety was then incorporated by coupling with the corresponding acid chloride, which was generated *in situ* using oxalyl chloride. The reduction of the nitro group in **51a** and **52a** by treatment with zinc in a mixture of acetic acid/methanol afforded final compounds **5a** and **6a**. The latter was reduced using lithium aluminum hydride to obtain final analogue **7a** (Scheme 11). Nevertheless, the reduction of amide **5a** under the same conditions did not work and a complex reaction mixture was obtained. Other conditions and reducing agents were investigated (Table 3) but none of them provided the corresponding amine compound.

Intermediates **39b** and **42b**, prepared following the cascade reaction described in Schemes 7 and 8 using the *R* catalyst, were derivatized according to Scheme 11 to give enantiomers **5b**-**7b**. Both enantiomers of final compounds **5**-**7** were obtained with high *ee* (94%).



Scheme 11. Reagents and conditions: (a) $NaClO_2$, KH_2PO_4, 2-methylbut-2-ene, *t*-BuOH/THF, 30 ºC, on, 79%-quantitative; (b) i. $(COCl)_2$ (2 M in DCM), cat. DMF, DCM, rt, 1 h; ii. (cyclopropylmethyl)amine, 0 ºC to rt, 3-18 h, 55%-quantitative; (c) Zn, AcOH/MeOH, rt, 4 h, 64-87%; (d) $LiAlH_4$, THF, reflux, 24 h, 20-22%.

Table 3. Experimental conditions for the amide reduction of compound **5a**



(2*S*,3*S*,4*S*)-**5** (**5a**)

Entry	Reducing agent	Solvent	Temp (ºC)	t (h)	Product
1	$LiAlH_4$	THF	reflux	on	CRM
2	$LiAlH_4$	THF	rt	on	SM and CRM
3	BH_3·THF	THF	rt	6	SM
4	BH_3·THF	THF	reflux	on	CRM
5	$NiCl_2$(dme) (10 mol%) / $PhSiH_3$	toluene	reflux	24	CRM

SM: starting material; CRM: complex reaction mixture.

2.5. Synthesis of pyridopyrazine scaffold. Final compound 10

1,2-dihydroquinolines can be assembled from 2-aminobenzaldehyde and an *α*,*β*-unsaturated aldehyde as Michael acceptor through an asymmetric domino *aza*-Michael-aldol reaction (X = CH, Figure 15).[72] Thus, we considered to apply this approach to obtain the dyhydropyrido[2,3-*b*]pyrazine scaffold (X = N, Figure 15) of target compound **10**, by reaction between 3-aminopyrazine-2-carbaldehyde (**53**) and *p*-methoxycinnamaldehyde.

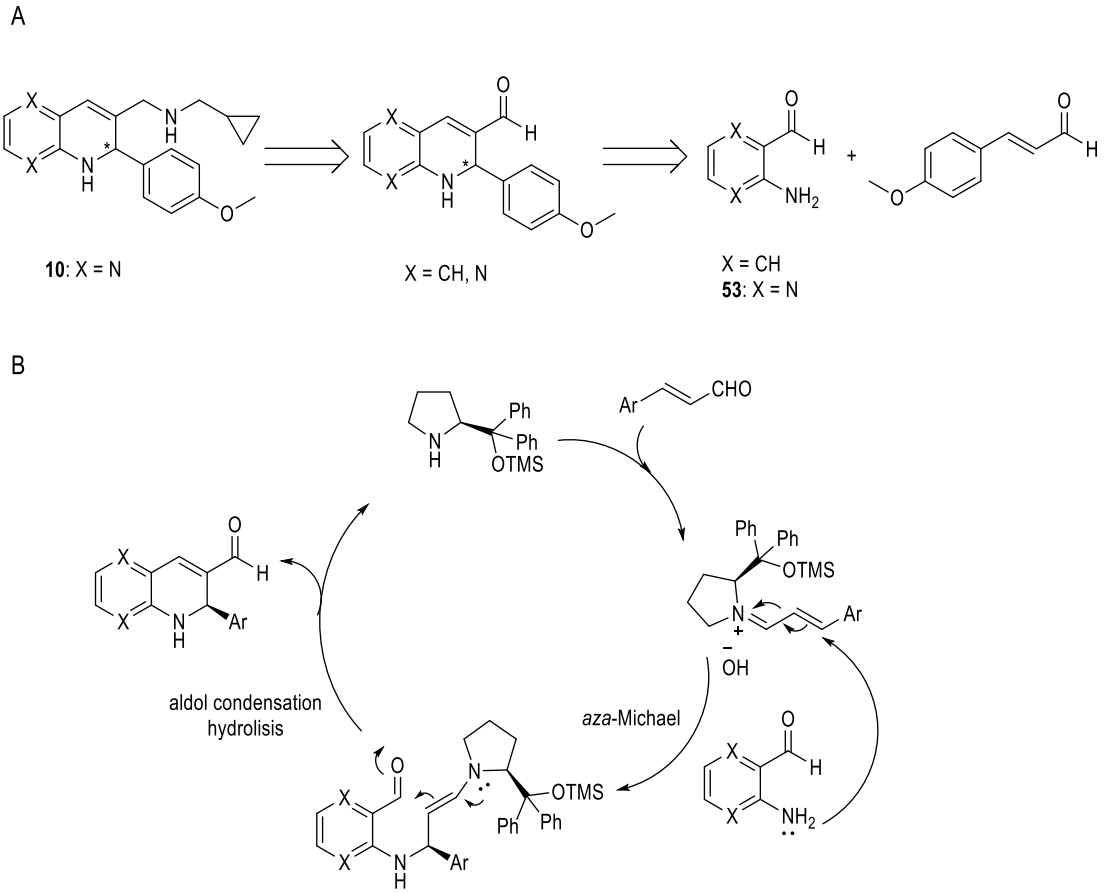
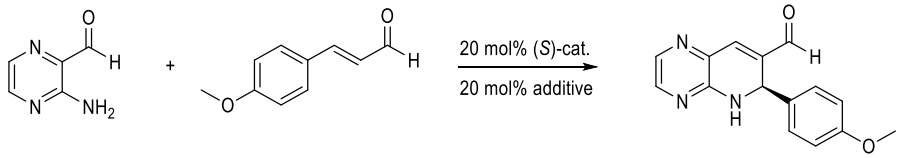


Figure 15. Synthetic approach for target compound **10**, containing a dyhydropyrido[2,3-*b*]pyrazine scaffold (X = N) (A) and proposed reported mechanism for the cascade reaction (B)

Compound **53** was synthesized by reduction of methyl 3-amino-2-pyrazinecarboxylate with DIBALH.[73] Next, the reaction of **53** and *p*-methoxycinnamaldehyde was carried out following the reported procedure, using the chiral prolinol derivative as catalyst and benzoic acid as additive at -25 ºC. However, after 7 days of reaction no product was detected by ^{1}H-NMR analysis of the reaction mixture (Table 4, entry 1). We explored different reaction conditions by changing the temperature, the solvent and the additive, but no reaction product was observed (Table 4, entries 2-4). Further increase of the reaction temperature above rt was discarded to avoid secondary reactions of **53**, considering it could compete with the catalyst for imine formation, which would lead to a racemic product or that the desired compound is not formed. The lack of reactivity of **53** in the domino reaction may be explained by the presence of the two nitrogens in the pyrazine ring, turning the amino group less nucleophilic. In view of these results,

we decided to test the experimental conditions used for the synthesis of the chromane derivative **39** (see Scheme 7). In this case, the reaction of **53** and *p*-methoxycinnamaldehyde using the *S* form of prolinol catalyst and acetic acid in chloroform at rt afforded compound **54a** after 14 days in 30% yield (Table 4, entry 6). Finally, an increase of the concentration of the reaction allowed us to obtain **54a** in 40% yield after 7 days (Table 4, entry 7).

Table 4. Experimental conditions for the domino reaction to obtain compound **54a**

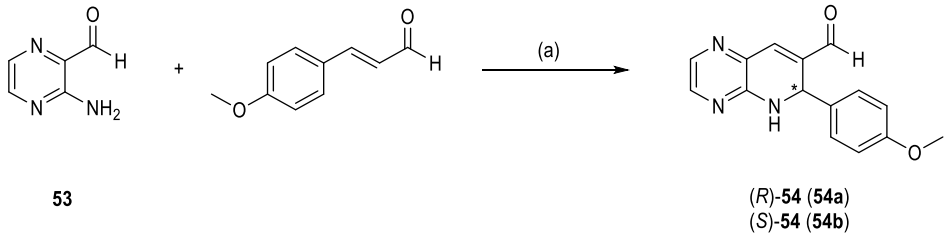


53 (*R*)-**54** (**54a**)

Entry	Additive	Scale[a] (mmol)	Molarity[b] (M)	Solvent	Temp (ºC)	t (days)	Yield (%)
1	benzoic acid	0.40	0.4	DMF	-25	7	-
2	benzoic acid	0.40	0.4	DMF	rt	7	-
3	benzoic acid	0.40	0.4	CH_3CN	rt	7	-
4	acetic acid	0.40	0.4	CH_3CN	rt	7	-
5	acetic acid	0.40	0.4	$CHCl_3$	rt	7	15
6	acetic acid	0.40	0.4	$CHCl_3$	rt	14	30
7	acetic acid	0.40	0.2	$CHCl_3$	rt	7	40
8	acetic acid	1.20	0.2	$CHCl_3$	rt	14	20

[a]Scale referenced to mmol of **53**; [b] Molarity referenced to **53**.

All the optimization reactions were performed on a 0.4-mmol scale (50 mg) of **53**, and unfortunately, an attempt to increase the reaction scale up to 1.2-mmol scale (150 mg) did not work, causing a significant increase in the reaction time (Table 4, entry 8). Hence, all the subsequent reactions to prepare enantiomers **54a** and **54b** were conducted on a 0.3-mmole scale (50 mg) of **53**, using the appropriate enantiomeric form of the prolinol catalyst (Scheme 12). The absolute configuration of **54a** and **54b** was established according to the stereochemical outcome of the previously reported organocascade reaction to afford 1,2-dihydroquinolines.[72]

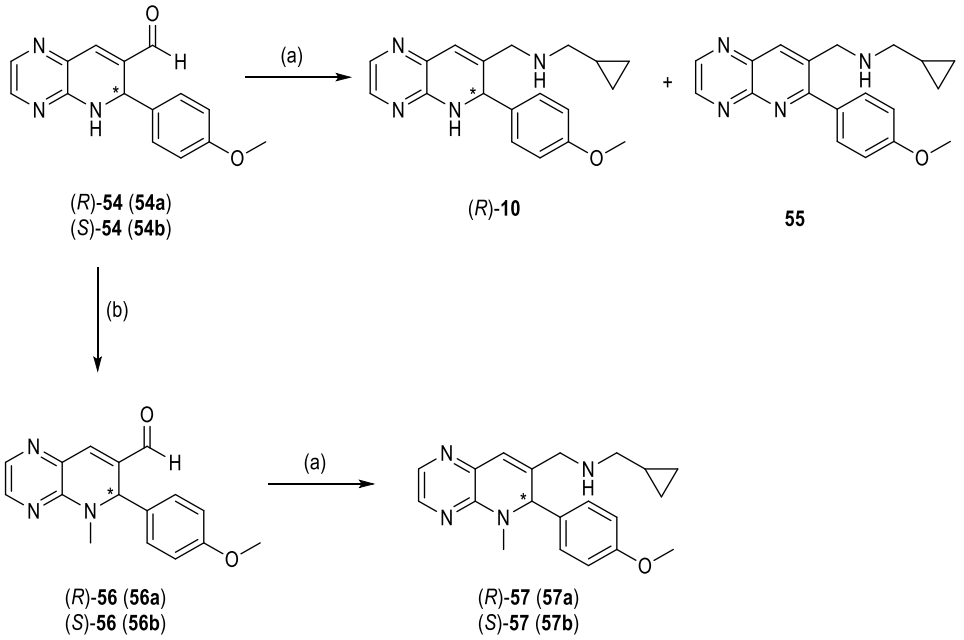


Scheme 12. Reagents and conditions: (a) 20 mol% chiral cat., 20% mol AcOH, $CHCl_3$, rt, 7 days, 40-45%.

Intermediate **54a** was derivatized via reductive amination, as described in Scheme 13. The reaction of **54a** with an excess of (cyclopropylmethyl)amine for 3 h and *in-situ* treatment with sodium borohydride provided final compound (*R*)-**10**, together with aromatic derivative **55** as secondary product. Both compounds were successfully isolated by flash chromatography, but (*R*)-**10** turned out to be unstable and evolved to derivative **55** at rt. This instability may be explained by the loss of the conjugation with the aldehyde group, which is then achieved through aromatization.

In order to obtain the designed dihydropyrido[2,3-*b*]pyrazine analogues, **54** was *N*-alkylated to block the aromatization after the reductive amination. Thus, **54a** was alkylated with methyl iodide using cesium carbonate as a base to yield **56a**. Then, the reductive amination of **56a** with (cyclopropylmethyl)amine afforded final compound **57a** that remained stable (Scheme 13).

Intermediate **54b**, synthesized via the cascade using the *R* form of the catalyst (Scheme 12), was transformed into enantiomer **57b** following an analogue synthetic route (Scheme 13). Both enantiomers of final compound **57** were obtained with high *ee* (98%).



Scheme 13. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH/DCM, rt, 3 h; ii. $NaBH_4$, 0 ºC to rt, 1-2 h, 80% for **10**, 10% for **55**, 49-50% for **57**; (b) CH_3I, Cs_2CO_3, DMF, rt, 1 h, 95%-quantitative.

2.6. Synthesis of tetrasubstituted cyclohexene scaffold. Final compounds 11-20

For the synthesis of final compounds **11**-**15** we proposed the use of a triple cascade organocatalytic reaction comprising an aliphatic aldehyde, a nitroalkene and an α,β-unsaturated aldehyde (Figure 16).[74] This three-component cascade reaction proceeds by way of a catalyzed Michael-Michael-aldol condensation sequence affording cyclohexenecarbaldehydes with moderate yields and excellent diastereo- and enantioselectivity (Figure 16). Thus, the organocatalytic reaction between propionaldehyde, *trans*-*p*-methoxy-*β*-nitrostyrene and acrolein using the corresponding *S* or *R* form of 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine as the catalyst provided trisubstituted cyclohexenecarbaldehyde intermediate **58a** or **58b**, respectively (Scheme 14). The reaction took place with high diastereoselectivity (dr = 9:1, determined by ^{1}H-NMR analysis of the reaction crude) and the major diastereoisomer was isolated in pure form by flash chromatography. The relative configuration in compound **58a** was assigned using NOE experiments. Thus, NOEs between the proton in α position to the methyl group and the aromatic protons confirmed the *trans* configuration for methyl and *p*-methoxyphenyl groups; the NOE between $CHNO_2$ proton and the proton in α

position to the aromatic ring indicated a relative *cis* disposition for nitro and *p*-methoxyphenyl groups. The absolute configuration of **58a** and **58b** was assigned according to the stereochemical outcome of the reported asymmetric organocatalytic reaction. [74]

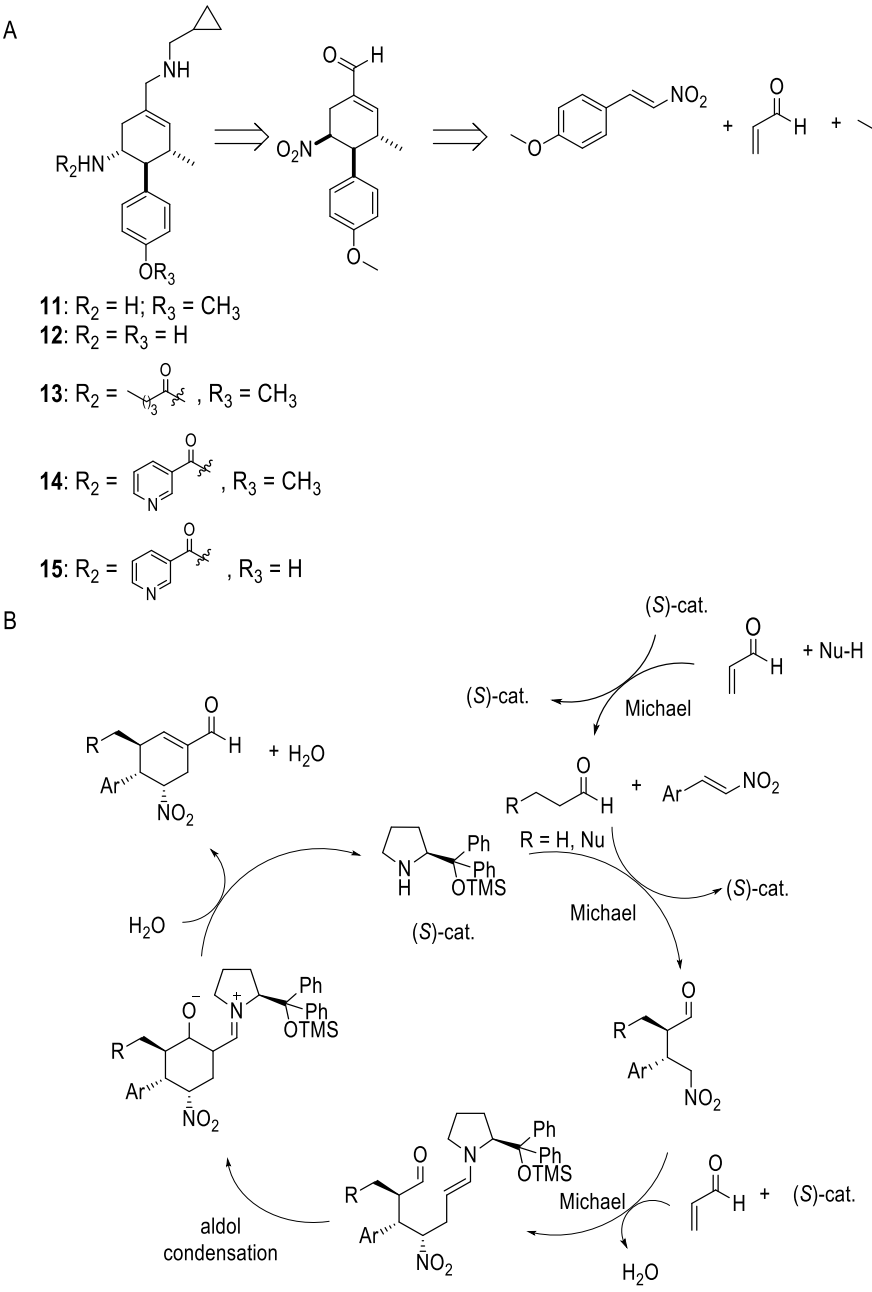
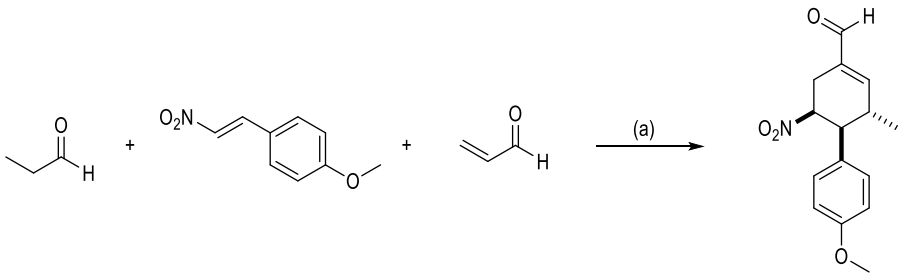


Figure 16. Synthetic approach for target compounds **11**-**15**, containing a tetrasubstituted cyclohexene scaffold (A) and proposed reported catalytic cycle of the triple or quadruple cascade (B)

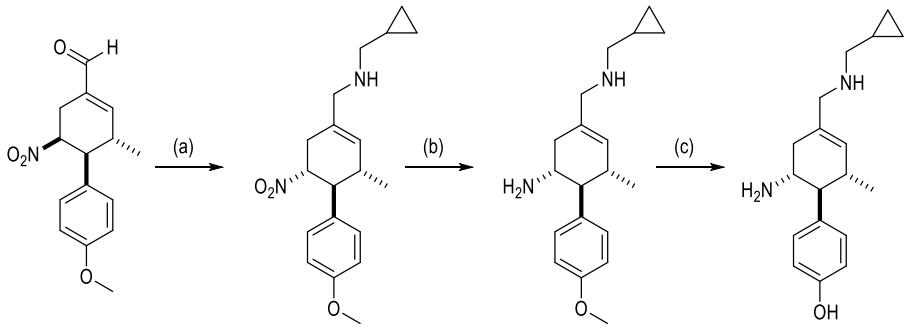


(3*S*,4*S*,5*S*)-**58** (**58a**)
(3*R*,4*R*,5*R*)-**58** (**58b**)

Scheme 14. Reagents and conditions: (a) 20 mol% chiral cat., toluene, 0 ºC, 1 h, rt, on, 50-59%.

Then, enantiomer cyclohexenecarbaldehyde **58a** was treated with an excess of (cyclopropylmethyl)amine followed by reduction *in situ* with sodium borohydride to obtain compound **59a** (Scheme 15). The NOE difference spectrum of **59a** indicated the complete inversion of the chiral centre adjacent to the nitro group, resulting in a favourable relative *trans* configuration of the aryl and the nitro group. Next, we carried out the reduction of nitro group using zinc in a mixture of acetic acid/methanol to afford final compound **11a** that was further demethylated to analogue **12a** using boron tribromide at 0 ºC (Scheme 15).

Compounds **11b** and **12b** were obtained from enantiomer **58b** following an analogue synthetic route (Scheme 15).



(3*S*,4*S*,5*S*)-**58** (**58a**)
(3*R*,4*R*,5*R*)-**58** (**58b**)

(3*S*,4*S*,5*R*)-**59** (**59a**)
(3*R*,4*R*,5*S*)-**59** (**59b**)

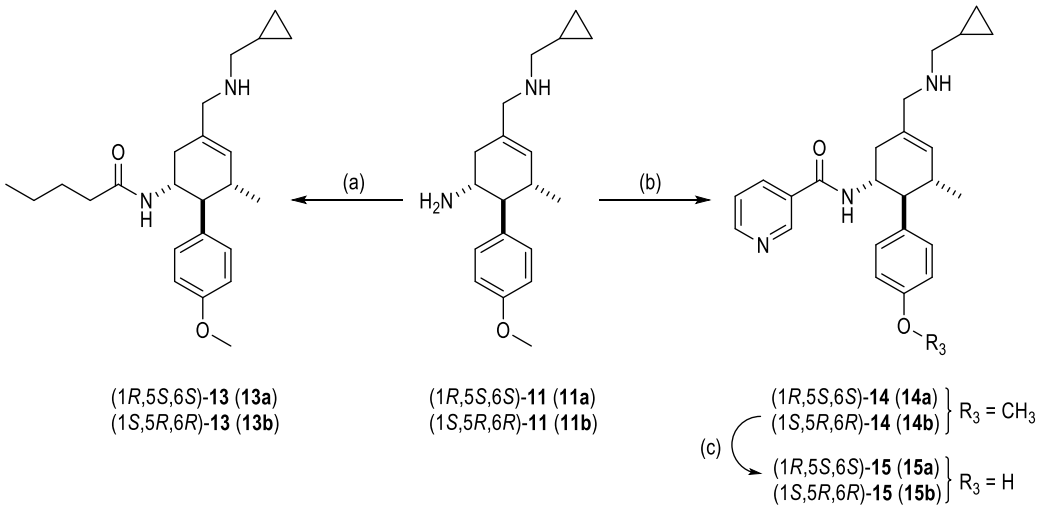
(1*R*,5*S*,6*S*)-**11** (**11a**)
(1*S*,5*R*,6*R*)-**11** (**11b**)

(1*S*,2*S*,6*R*)-**12** (**12a**)
(1*R*,2*R*,6*S*)-**12** (**12b**)

Scheme 15. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH, rt, 3 h; ii. $NaBH_4$, rt, 2 h, 57-74%; (b) Zn, AcOH/MeOH, 1 h, 90-94%; (c) BBr_3, DCM, 0 ºC, 1 h, 58-65%.

In tetrasubstituted cyclohexene derivatives **13** and **14**, a fatty-acid short chain or a nicotinic acid moiety were introduced as an additional PrSc present in microbiota metabolites. **13a** or **14a** were prepared by coupling reaction of amine **11a** with valeric or nicotinic acid, respectively, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBt) as coupling agents, and *N*,*N*-diisopropylethylamine (DIPEA) as base. The low yield in the formation of these amides was due to the formation of diamide compound as secondary product. Demethylation of **14a** using boron tribromide at 0 ºC afforded analogue **15a** (Scheme 16).

The corresponding enantiomers **13b**-**15b** were obtained from the free amine **11b** using the same synthetic pathway (Scheme 16). Final cyclohexene compounds **13**-**15** were obtained with *ee* higher than 99%.



Scheme 16. Reagents and conditions: (a) valeric acid, EDC, HOBt, DIPEA, DCM, 0 ºC, 2 h, 18-21%; (b) nicotinic acid, EDC, HOBt, DIPEA, DCM, 0 ºC, 2 h, rt, 2 h, 25-36%; (c) BBr_3, DCM, 0 ºC, 1 h, 40-42%.

In the case of tetrasubstituted derivatives **16-20** containing an indole or a thiazole as an additional microbiota PrSc, we applied a four-component cascade reaction starting from acrolein, nitroalkene and a nucleophilic compound using an enantiopure prolinol derivative (Figure 17).[75]

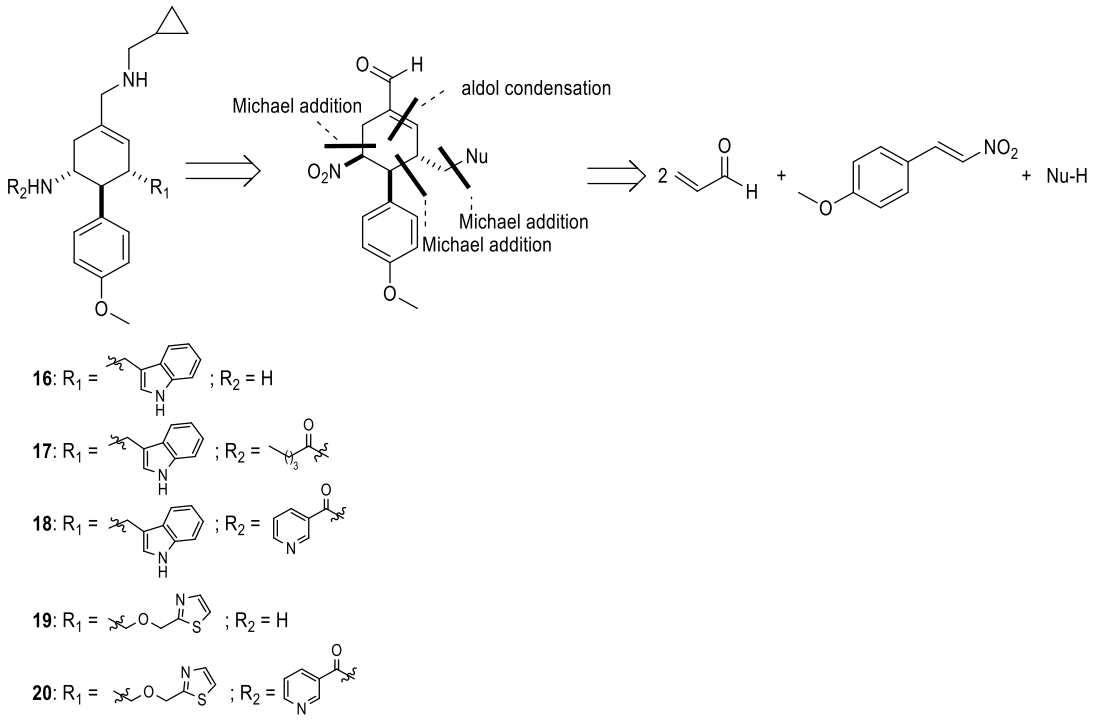
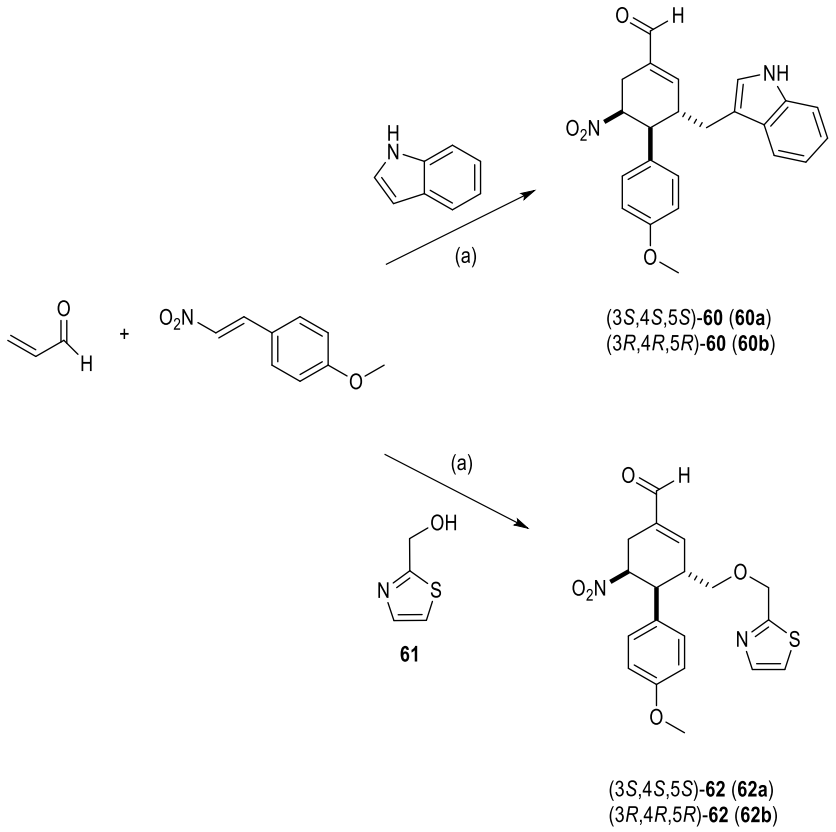


Figure 17. Synthetic approach for target tetrasubstituted cyclohexenes **16**-**20** via a four-component cascade reaction

This reaction is a quadruple domino Michael-Michael-Michael-aldol condensation reaction that proceeds in a diastereo- and enantioselective manner. The cascade is initiated by Michael addition of the nucleophile after an iminium activation of acrolein, followed sequentially by an enamine- and an iminium-mediated Michael additions, to finish with an intramolecular aldol-condensation to form the cyclohexenecarbaldehyde core (Figure 16). The addition of acrolein must be done slowly to avoid a polymerization side reaction, which may be attributed to a competing Michael addition with acrolein itself as acceptor.

Thus, the reaction between acrolein, *trans*-*p*-methoxy-*β*-nitrostyrene and indole as nucleophile in chloroform, using the *S* or *R* form of 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine as catalyst, afforded compound **60a** or **60b**, respectively (Scheme 17). When alcohol **61** was used as nucleophile, using the corresponding *S* or *R* form of the catalyst, intermediates **62a** or **62b** were obtained, respectively. Alcohol **61** was previously synthesized by reduction of 1,3-thiazol-2 carbaldehyde with sodium borohydride.

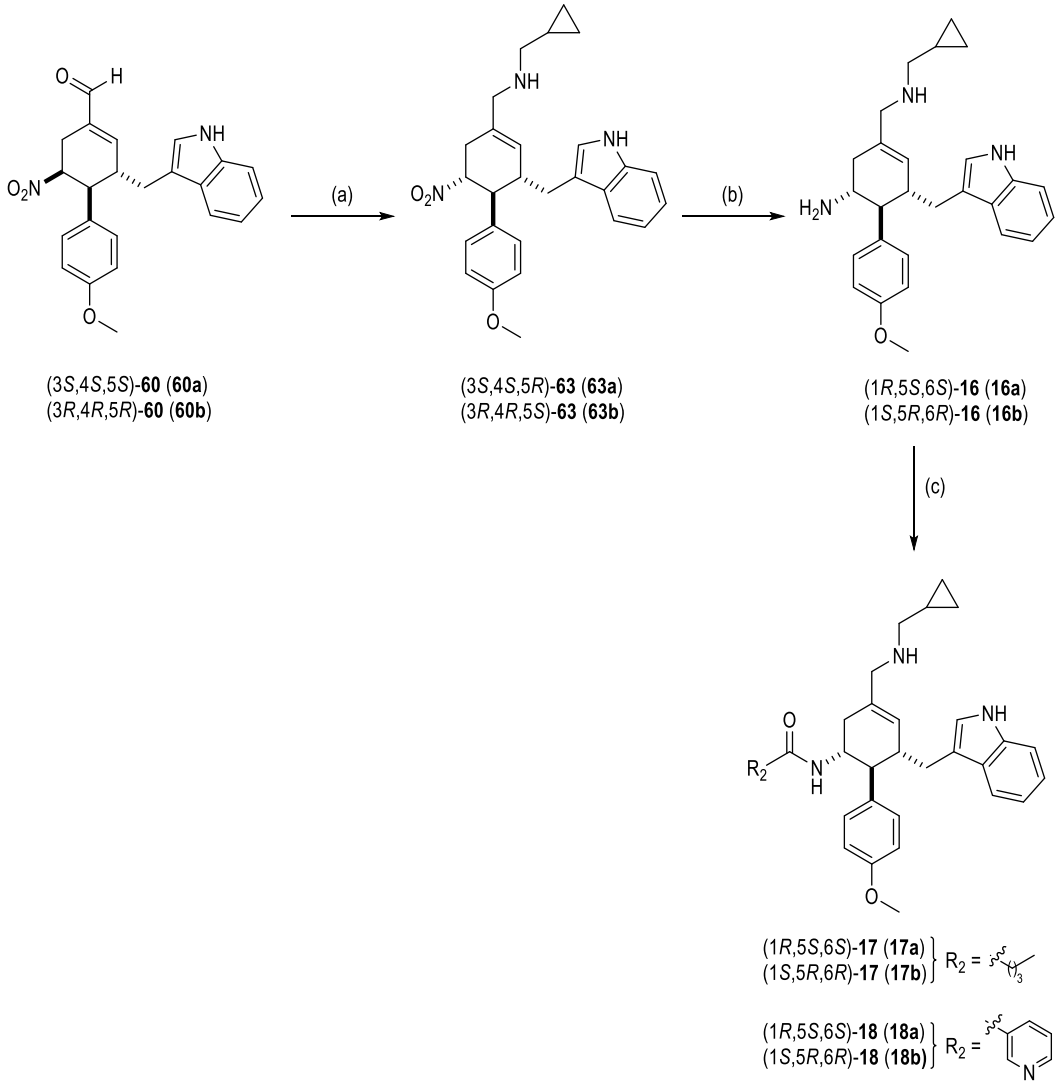
Both compounds **60** and **62** were formed with high diastereoselectivity (dr = 95:5, determined by ^{1}H-NMR analysis of the reaction crude), and the major diastereoisomer was isolated in pure form by column chromatography. The relative configuration of the compound is analogous to that of the previous cyclohexene derivative **58** and it was determined by NOE experiments. The absolute configuration of enantiomers of **60** and **62** was assigned according to the stereochemical outcome of the reported asymmetric organocatalytic cascade.[75]



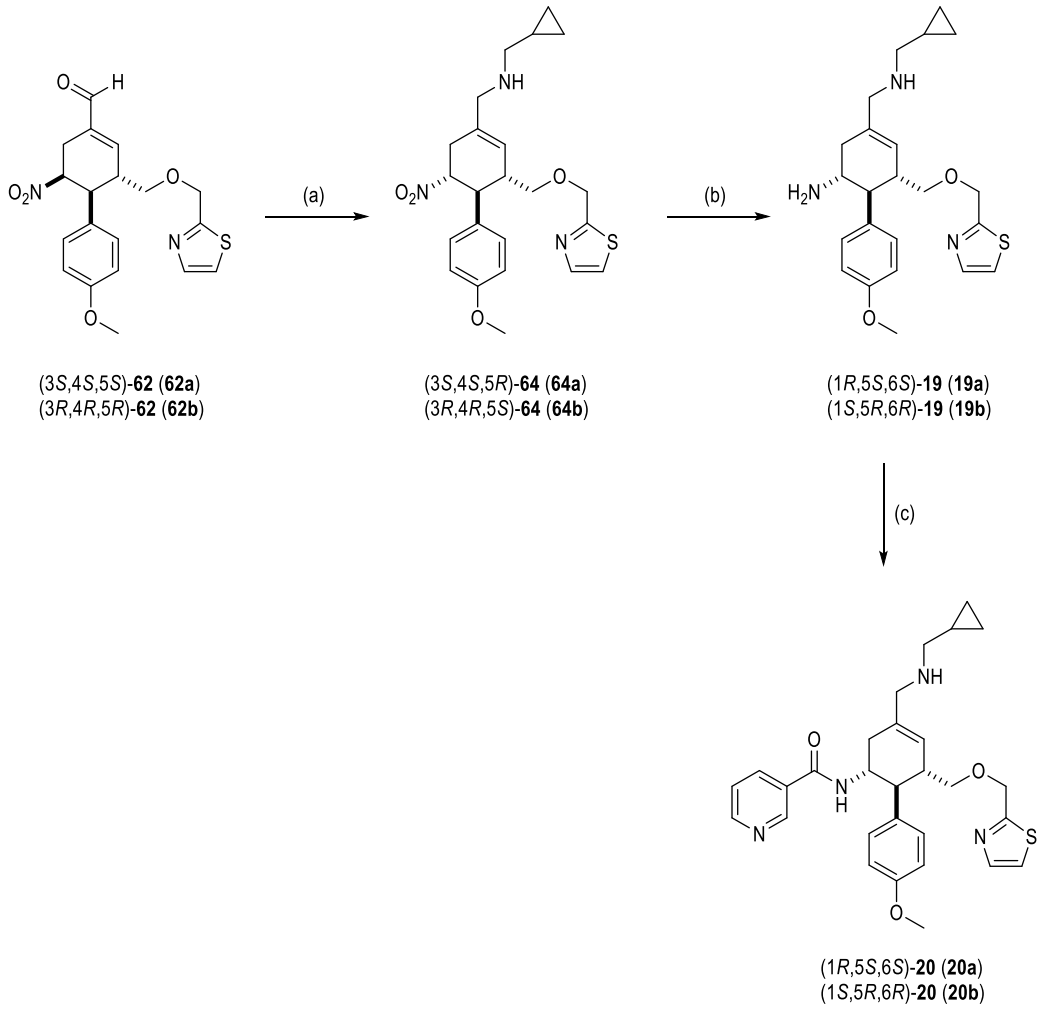
Scheme 17. Reagents and conditions: (a) 10 mol% chiral cat., $CHCl_3$, rt., 24-40 h, 49-54%.

Intermediates **60a** and **62a** were derivatized using the sequence of reductive amination and reduction of the nitro group, to obtain final compounds **16a** and **19a**, respectively (Schemes 18 and 19). Complete inversion of the chiral center adjacent to the nitro group was observed resulting in a favourable relative *trans* configuration for *p*-methoxyphenyl group and the other two substituents in the cyclohexene. Finally, valeric or nicotinic acid moieties were introduced via coupling reaction using EDC, HOBt and DIPEA, to afford analogues **17a**, **18a** and **20a** (Schemes 18 and 19).

Intermediates **60b** and **62b**, obtained in the cascade reaction using the *R* form of catalyst (Scheme 17), were transformed into enantiomers **16b**-**20b** following an analogue synthetic route (Schemes 18 and 19). Both enantiomers of each final compound **16-20** were obtained with excellent ee (99%).



Scheme 18. Reagents and conditions: (a) i. (cyclopropylmethyl)amine, MeOH, rt, 3 h; ii. $NaBH_4$, rt, 3 h, 82-87%; (b) Zn, AcOH/MeOH, 1 h, 87-95%; (c) valeric or nicotinic acid, EDC, HOBt, DIPEA, DCM, 0 ºC, 2-3 h, rt, 1-2 h, 13-30%.



(3*S*,4*S*,5*S*)-**62** (**62a**)
(3*R*,4*R*,5*R*)-**62** (**62b**)

(3*S*,4*S*,5*R*)-**64** (**64a**)
(3*R*,4*R*,5*S*)-**64** (**64b**)

(1*R*,5*S*,6*S*)-**19** (**19a**)
(1*S*,5*R*,6*R*)-**19** (**19b**)

(1*R*,5*S*,6*S*)-**20** (**20a**)
(1*S*,5*R*,6*R*)-**20** (**20b**)

Scheme 19. Reagents and conditions: (a) i) (cyclopropylmethyl)amine, MeOH, rt, 3 h, ii) $NaBH_4$, rt, 2 h, 66-76%; (b) Zn, AcOH/MeOH 1:1, rt, 1 h, 78-93%; (c) nicotinic acid, EDC, HOBt, DIPEA, DCM, 0 ºC, 2 h, 23-30%.

2.7. *In-vitro* CSC model: Cellular phenotypic screening based on cancer cell differentiation

New synthesized compounds **1**-**9**, **11**-**20**, **46**-**48**, **55**, and **57**, bearing PrSc present in microbiota metabolites, were screened in cellular phenotypic assays aimed to assess their ability to regulate the differentiation process in cancer models. Toward this end, we proposed to study the tumorsphere formation, as a characteristic growth in CSCs, in the presence of the compounds at a non-cytotoxic concentration in non-stem tumor cells which was determined by MTT assays.

First, the cytotoxicity of the new compounds in breast cancer MCF-7 cell line, as well as in colon cancer HCT-116 cell line, was evaluated after 48 h of incubation with tested compound at 10 µM, using a colorimetric assay based on the metabolic reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Figure 18A). Remaining viable cells are able to reduce MTT to purple formazan crystals, which are quantified by measuring their absorbance at 570 nm relative to the vehicle-treated cells. Compounds exhibing a cytotoxicity higher than 35% were assayed at lower concentrations (5, 1, 0.5, 0.1 µM) to determine the non-toxic concentration. The obtained data in tumor cells are shown in Table 6.

The resulting non-toxic concentration was then used in the sphere-formation assay to ensure the observed effect is due to the regulation of the differentiation process in CSCs. Hence, MCF-7 and HCT-116 tumor cells were cultured in non-adherent and serum-free conditions in order to enrich CSCs. In these conditions non-stem cells are not able to survive, being CSCs the only viable ones, which grow grouped in form of spheres. After 10 days, the formation of mammospheres and colonspheres was visualized using confocal laser or phase contrast microscopy (Figure 18B).

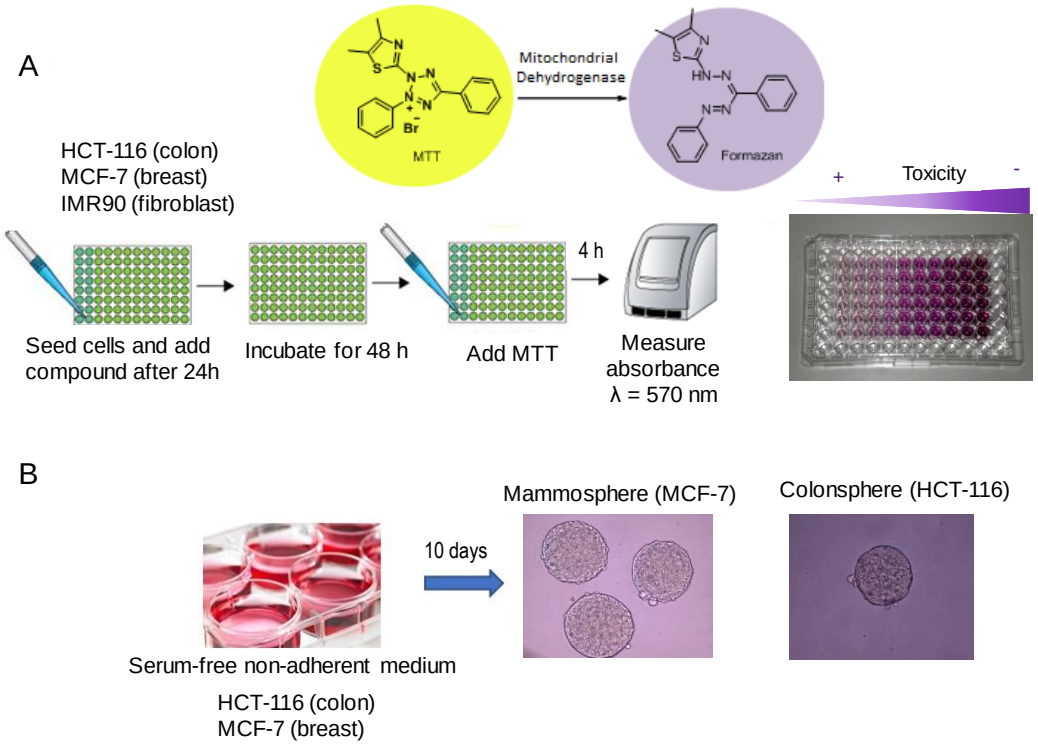


Figure 18. MTT assay to evaluate the cytotoxicity of tested compound in cell lines (A). Mammospheres and colonspheres formed after 10 days in non-treated cells under serum-free and non-adherent conditions, visualized using phase contrast microscopy (B)

For the screening, tested compound was added at seeding at the non-toxic concentration previously determined in the MTT assay. After 10 days of incubation, the resulting mammospheres and colonspheres were quantified. The obtained results are expressed in Table 6, and representative microscope images for active and inactive compounds are shown in Figure 19.

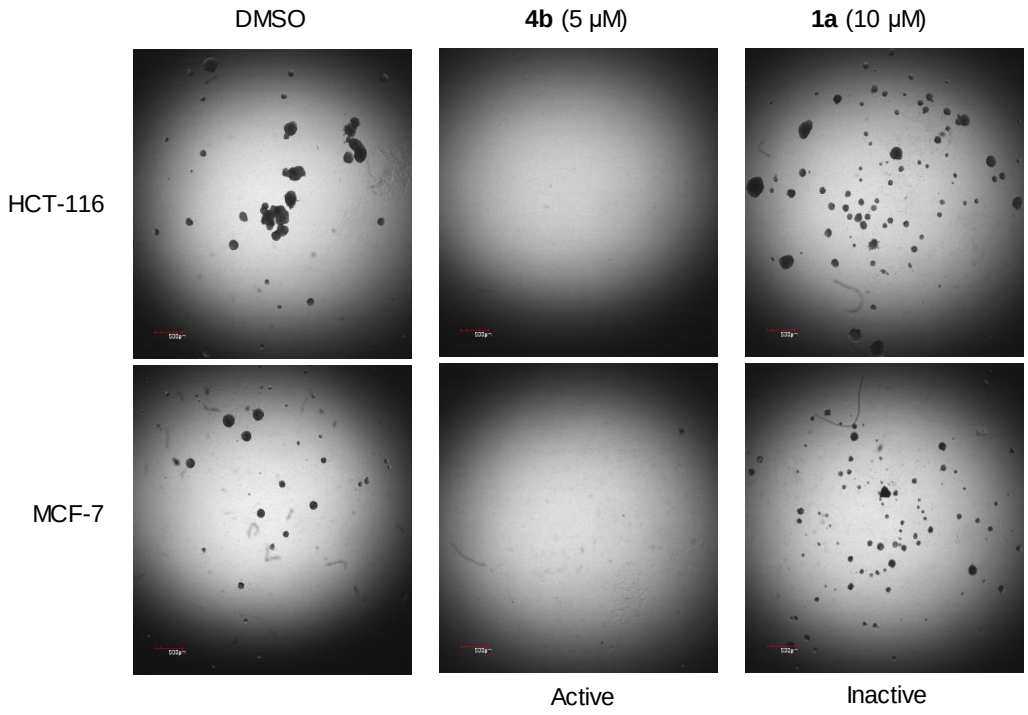


Figure 19. Representative active (**4b**) and inactive (**1a**) compounds in tumorsphere-formation assay in breast (MCF-7) and colon (HCT-116) cancer cell lines. Images were obtained using confocal laser microscopy. Scale bar, 500 μm

Table 6. Phenotypic screening data of final compounds in breast (MCF-7) and colon (HCT-116) cancer cell lines[a]

Compd	Cytotoxicity (%)		Inhibition of tumorsphere-formation (%)	
	MCF-7 (@ 10 μM)	HCT-116 (@ 10 μM)	MCF-7 (@ 10 μM)	HCT-116 (@ 10 μM)
1a	9 ± 7	30 ± 2	0	0
1b	20 ± 2	3 ± 2	0	0
2a	4 ± 3	10 ± 1	0	0
2b	3 ± 3	6 ± 3	0	0
3a	2 ± 2	5 ± 1	0	0
3b	5 ± 4	6 ± 3	0	0
4a	8 ± 7[c]	15 ± 2[e]	0[c]	0[e]
4b	4 ± 2[b]	30 ± 3[b]	100[b]	100[b]
5a	11 ± 6	39 ± 8	4 ± 2	2 ± 1
5b	6 ± 5	30 ± 1	0	0
6a	8 ± 2	6 ± 2	0	0
6b	1 ± 1	6 ± 2	0	0
7a	10 ± 2	10 ± 4	0	0
7b	27 ± 3	8 ± 3	0	0
8a	1 ± 1[c]	13 ± 1[c]	100[c]	100[c]
8b	13 ± 1[c]	6 ± 6[c]	100[c]	100[c]
9a	6 ± 11[b]	19 ± 6[c]	100[b]	100[c]
9b	6 ± 5[b]	22 ± 9[c]	100[b]	100[c]
11a	35 ± 6	13 ± 7	100	100
11b	34 ± 4	27 ± 2	100	100
12a	40 ± 1	6± 6	0	33 ± 7
12b	2 ± 1	7 ± 2	0	51 ± 2
13a	46 ± 1	0 ± 0	23 ± 4	81 ± 2
13b	14 ± 8	4 ± 4	0	37 ± 5
14a	32 ± 2	12 ± 2	43 ± 7	30 ± 6
14b	2 ± 2	0 ± 0	100	100
15a	0 ± 0	14 ± 3	0	0
15b	19 ± 13	12 ± 12	0	0
16a	0[c]	16 ± 4[c]	78 ± 5[c]	100[c]
16b	0[c]	15 ± 2[c]	100[c]	100[c]
17a	8 ± 6[b]	24 ± 4[b]	100[b]	100[b]
17b	20 ± 10[b]	26 ± 3[b]	100[b]	100[b]
18a	7 ± 7[b]	30 ± 13[b]	100[b]	100[b]
18b	0 ± 0[b]	26 ± 1[b]	100[b]	100[b]
19a	0 ± 0	31 ± 21	100	100
19b	0 ± 0	20 ± 15	100	100
20a	31 ± 2	19 ± 2	70 ± 10	44 ± 4
20b	0	0	100	100
46a	2 ± 1	8 ± 1	0	0
46b	4 ± 2	5 ± 2	0	0
47a	35 ± 1	6± 6	0	0
47b	2 ± 1	7 ± 2	0	0
48a	27 ± 5	6 ± 2	0	0
48b	35 ± 2	10 ± 3	0	0
55	32 ± 4	29 ± 3	0	0
57a	23 ± 8	15 ± 7	0	0
57b	11 ± 6	10 ± 3	0	0

[a]Data from two to three independent experiments performed in triplicate; [b]@5 μM; [c]@1 μM; [d]@0.5 μM; [e]@0.1 μM.

Those compounds able to inhibit the formation of breast and colon tumorspheres (inhibition >90% relative to vehicle-treated cells) with no cytotoxicity in the corresponding non-stem cells were considered to act on CSCs by promoting differentiation and/or inducing their death. Hence, three different microbiota-metabolite based scaffolds were identified in active compounds: tetrahydrocarbazole in compound **4b**, tetrahydrobenzo[*c*]chromene in analogues **8** and **9**, and tetrasubstituted cyclohexene in derivatives **11**, **14b**, **16**-**19**, and **20b** (Figure 20).

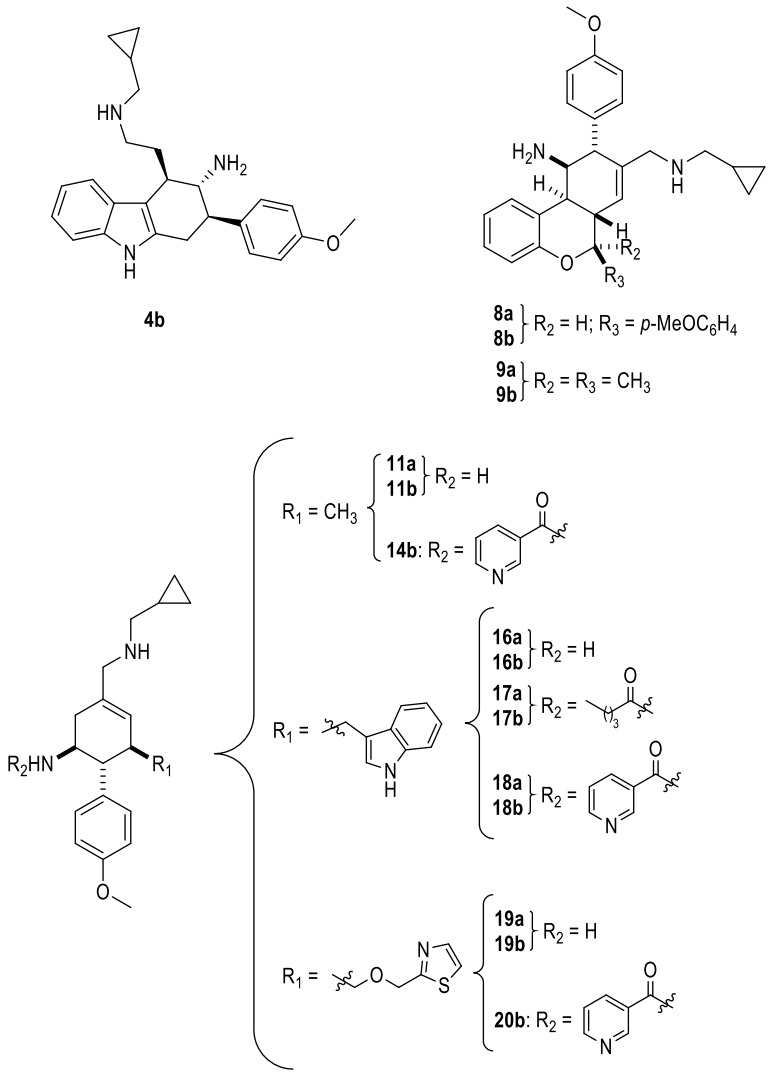


Figure 20. Microbiota-metabolite based compounds acting at CSCs

Next, in order to determine if the compounds are able to act on CSCs without affecting the viability of non-tumor cells, MTT cytotoxicity assay was performed in the fibroblast IMR90 cell line. From the data in Table 7, tetrahydrocarbazole **4b** was not toxic in non-tumor cells at 5 μM, while tetrahydrobenzo[*c*]chromenes **8a**, **8b**, **9a**, and **9b** exhibited the highest toxicity (43-80% @5 μM). As a general trend in tetrasubstituted cyclohexenes, cytotoxicity was higher in derivatives bearing an indole ring in R_1 moiety (e.g. **16a**, **16b**: 46%, 86% @5 μM) than in analogues that contain a methyl group or a thiazole ring in R_1. Indeed, compounds **11a**, **11b**, **14b**, **19a**, **19b**, and **20b** displayed no significant cytotoxicity (<14%) at the highest concentration of 10 μM.

Table 7. Cytotoxicity of compounds acting on CSCs in non-tumor fibroblast cell line[a]

Compd	Cytotoxicity (%) IMR90 (@ 5 μM)	Compd	Cytotoxicity (%) IMR90 (@ 5 μM)
4b	0	**16a**	46 ± 16 8 ± 6[b]
8a	80 ± 8 17 ± 6[b]	**16b**	86 ± 5 12 ± 6[b]
8b	72 ± 11 2 ± 2[b]	**17a**	32 ± 4
9a	47 ± 15 5 ± 5[b]	**17b**	25 ± 7
9b	43 ± 15 0[b]	**18a**	7 ± 3
11a	0[c]	**18b**	0
11b	5 ± 5[c]	**19a**	14 ± 11[c]
14b	0[c]	**19b**	3 ± 1[c]
		20b	5 ± 5[c]

[a]Data from two to three independent experiments performed in triplicate; [b]@1 μM; [c]@10 μM.

Taken together the results obtained from the cellular phenotypic screening, compounds **4b**, **11**, **14b**, **18**, **19**, and **20b** exhibited inhibition of tumorsphere formation and no cytotoxicity in tumor and non-tumor cells (Tables 6 and 7). Further biological evaluation was performed using *ex-vivo* samples from human patients, which are not readily available. Hence, **4b** (UCM13369) and **14b** (UCM13218) (see Figure 20) were first selected as representative analogues of tetrahydrocarbazole and tetrasubstituted cyclohexene scaffolds, respectively.

2.8. *Ex-vivo* CSC model: Study of selected compounds in blood samples from AML patients

Blood samples of AML patients were chosen as an *ex-vivo* CSC model to test the therapeutic potential of the selected compounds. As the largest concentration of hematopoietic stem cells is found in the bone marrow, healthy or AML patients were subjected to a surgical procedure to extract bone marrow from their rear pelvic bone. In collaboration with Hospital 12 de Octubre, whole mononuclear cells were isolated through Ficoll centrifugation method that takes advantage of the density differences between mononuclear cells and other elements found in the blood samples. At present, the most commonly referenced method for identifying cell types is based on the use of stem cell markers. The majority of these markers, which appear on the surface of the cells and play a role in cell signalling pathways or cell-cell adhesion molecules, are known as “cluster of differentiation” (CD), and are found specifically or commonly for the cell type. Immunomagnetic sorting utilizes antibodies against these surface markers to separate specific cells. This method was used to isolate $CD34^+$ hematopoietic stem cells, which were then used to perform colony-forming unit (CFU) assays to determine whether selected compounds functionally affect leukemic stem cells.

CFU is one of the most widely used assay for the study of hematopoietic stem cells. This assay allows measurement of the proliferation and differentiation ability of individual cells within a sample. Colonies are formed during the early myeloid differentiation of hematopoietic stem cells. In a semisolid medium that contains cytokines, about 14 days of culture are sufficient for the colonies to grow to a size that allows accurate counting by phase contrast microscopy. The number of colonies formed from a fixed number of input cells provides preliminary information about the ability of progenitors to differentiate, since the colonies represent the progeny of a single progenitor. In addition, the number of viable cells harvested from these colonies can be quantified as a measure of the proliferation ability of the hematopoietic stem cells.

To test the compounds in CFU assay, $CD34^+$ hematopoietic stem cells of leukemia patients were seeded in the absence or presence of different concentrations of selected compounds UCM13369 and UCM13218. The number of colonies was scored after 14 days and compared to vehicle-treated sample. As shown in Figure 21 (black bars), the compounds significantly decreased the formation of colonies derived from AML $CD34^+$ cells in the μM range and in a dose-dependent manner (IC_{50} < 5 μM). This result indicates that the compounds induce the death of stem $CD34^+$ cells and/or promote the differentiation to blood cells, losing their clonal ability. In addition, a decrease in the number of viable cells was

observed in the presence of both compounds (Figure 21, grey bars), which supports their effect in the proliferation of AML hematopoietic cells.

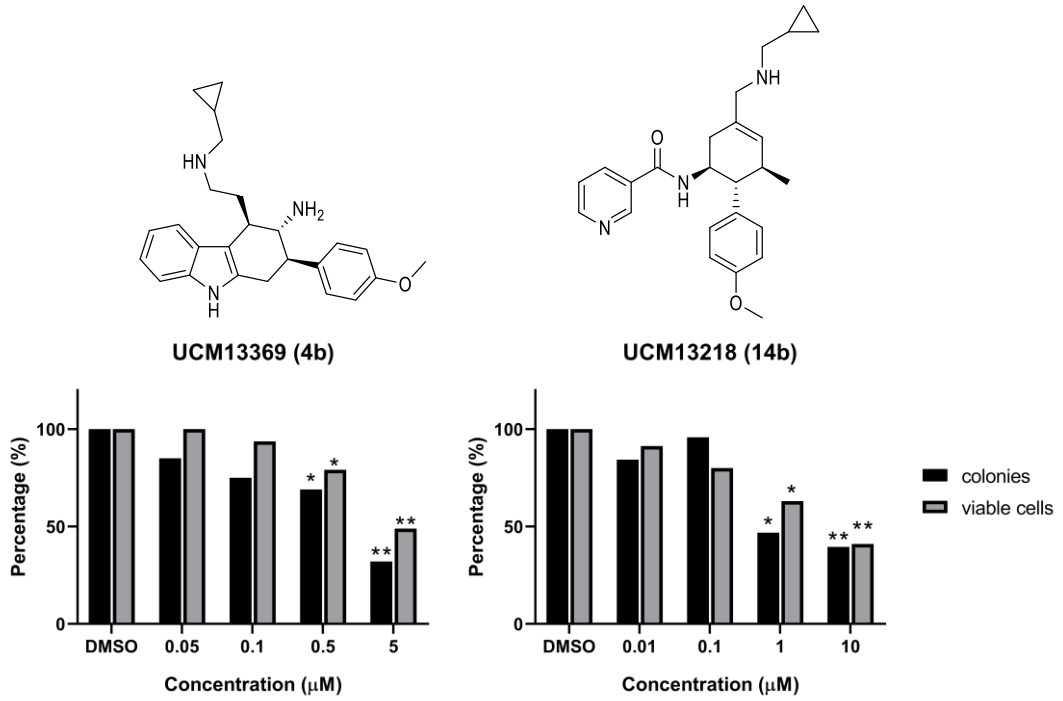


Figure 21. Colony-forming unit (CFU) assay in AML CD34$^+$ cells. The number of colonies and viable cells were determined using phase contrast microscopy in the absence or presence of selected compounds UCM13369 (**4b**) and UCM13218 (**14b**). All assays were performed in duplicate ($p<0.05$ *; <0.01 **)

Next, the cells from colonies were studied by flow cytometry. The cell population was analysed using CD34 antibody as stemness marker, CD71 as erythroblast (precursor of erythrocyte) marker, and CD45 as leukocyte marker (Figure 22). Also, annexin-V was employed to determine the cellular apoptotic population. The analysis of the stemness marker (CD34$^+$) was not conclusive and no difference was observed in the absence or presence of the compounds (data not shown), probably due to stemness disappearance after several days of culture. The results of CD71$^+$, CD45$^+$, and annexin-V$^+$ populations are shown in Figure 23.

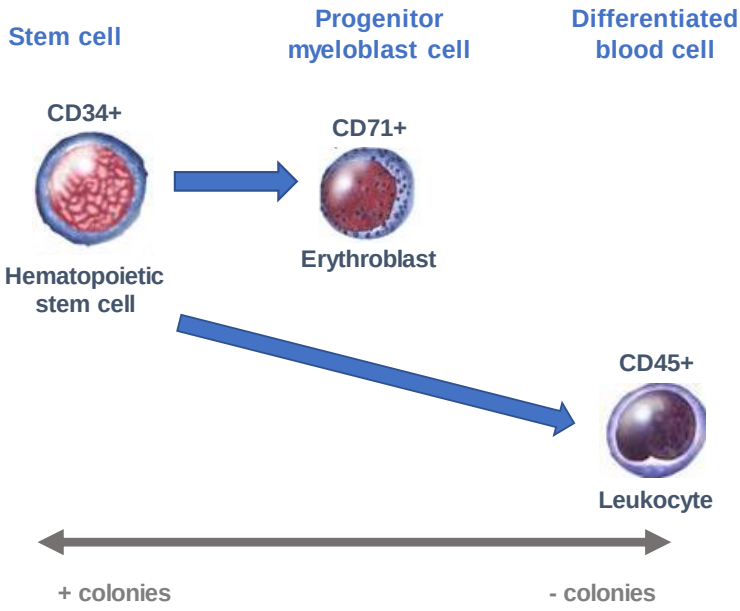


Figure 22. Specific markers of stem (hematopoietic stem cell), progenitor (erythroblast) and differentiated (leukocyte) cells

As AML is characterized by the accumulation of progenitor myeloblasts, patient samples display a higher $CD71^+$ erythroblast population (80%) than differentiated $CD45^+$ leukocytes (10%) (Figure 23A). This ratio was maintained in the presence of compound UCM13218, while after the administration of analogue UCM13369, $CD71^+$ population decreased to 54% and $CD45^+$ population increased to 41%. On the other hand, both compounds increased the apoptosis of unmature $CD71^+$ cells (Figure 23B). Notably, differentiated $CD45^+$ cells were not affected by treatment with the compounds (Figure 23C). Hence, UCM13218 and UCM13369 induce cell death of unmatured cells ($CD71^+$), while UCM13369 also promotes stem cell ($CD34^+$) differentiation to leukocytes ($CD45^+$). These results confirm the data previously obtained in the CFU and viability assays, and support the effect of the new compounds in the regulation of the differentiation and/or death of AML stem cells.

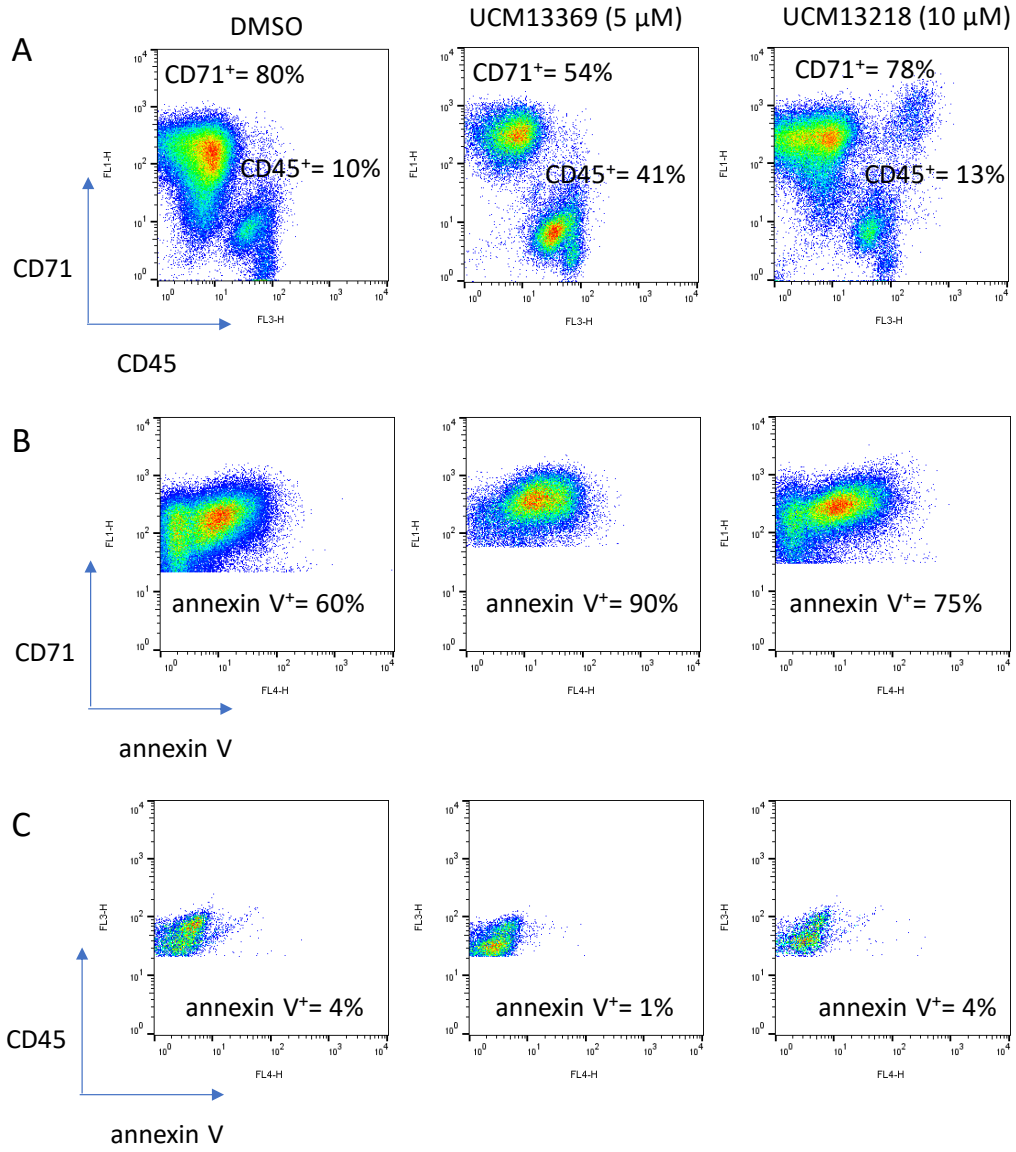


Figure 23. Flow cytometry analysis of $CD71^+$-$CD45^+$ (A), $CD71^+$-annexin V^+ (B), and $CD45^+$-annexin V^+ (C) populations after 14 days of treatment of AML $CD34^+$ cells with UCM13369 (5 µM) and UCM13218 (10 µM)

In addition, the effect of compounds UCM13369 and UCM13218 was studied in blood samples from healthy patients. Thus, isolated $CD34^+$ cells were grown in colonies, and in this case the compounds induced a lower decrease in CFU and cell viability (Figure 24) compared to AML patients (Figure 21).

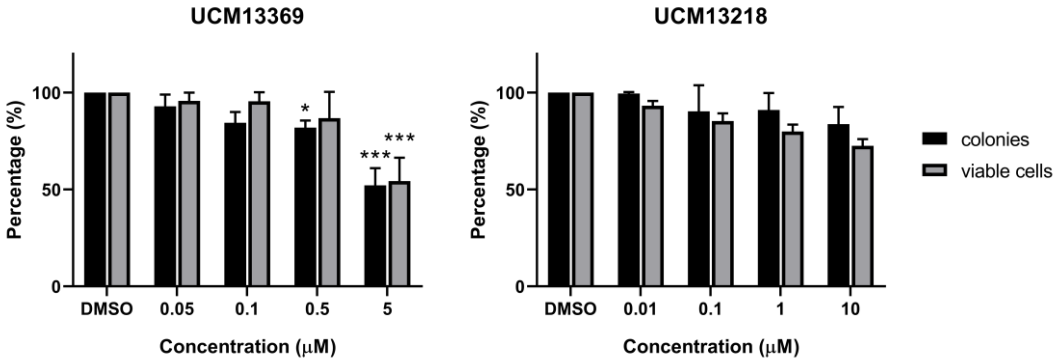


Figure 24. Colony-forming unit (CFU) assay in healthy CD34$^+$ cells. The number of colonies and viable cells were determined using phase contrast microscopy in the absence or presence of selected compounds UCM13369 and UCM13218. Data from four independent experiments performed in duplicate ($p<0.05$ *; <0.001 ***)

On the other hand, the analysis by flow cytometry of the non-treated cells derived from the colonies showed a smaller CD71$^+$ population than in AML patients (51 *vs* 71%, Figures 25A and 23A, respectively), as expected for healthy cells that maturate to erythrocytes. When healthy CD34$^+$ cells were incubated with UCM13369, CD71$^+$ population decreased to 20% and CD45$^+$ leukocyte population increased to 53%, while UCM13218 did not affect to CD71$^+$/CD45$^+$ ratio. Both compounds induced the apoptosis in CD71$^+$ cells, but not in CD45$^+$ cells (Figures 25B and 25C), being this effect more pronounced for UCM13369 where CD71$^+$-annexin V$^+$ increased from 30% in non-treated cells to 77%.

Altogether, the new compounds identified in this work, UCM13369 and UCM13218, exhibit a higher effect in the differentiation and/or death of AML stem cells than in healthy patient cells. Moreover, both compounds act on stem or progenitor cells without affecting differentiated cells, confirming that they are able to regulate the differentiation process in AML.

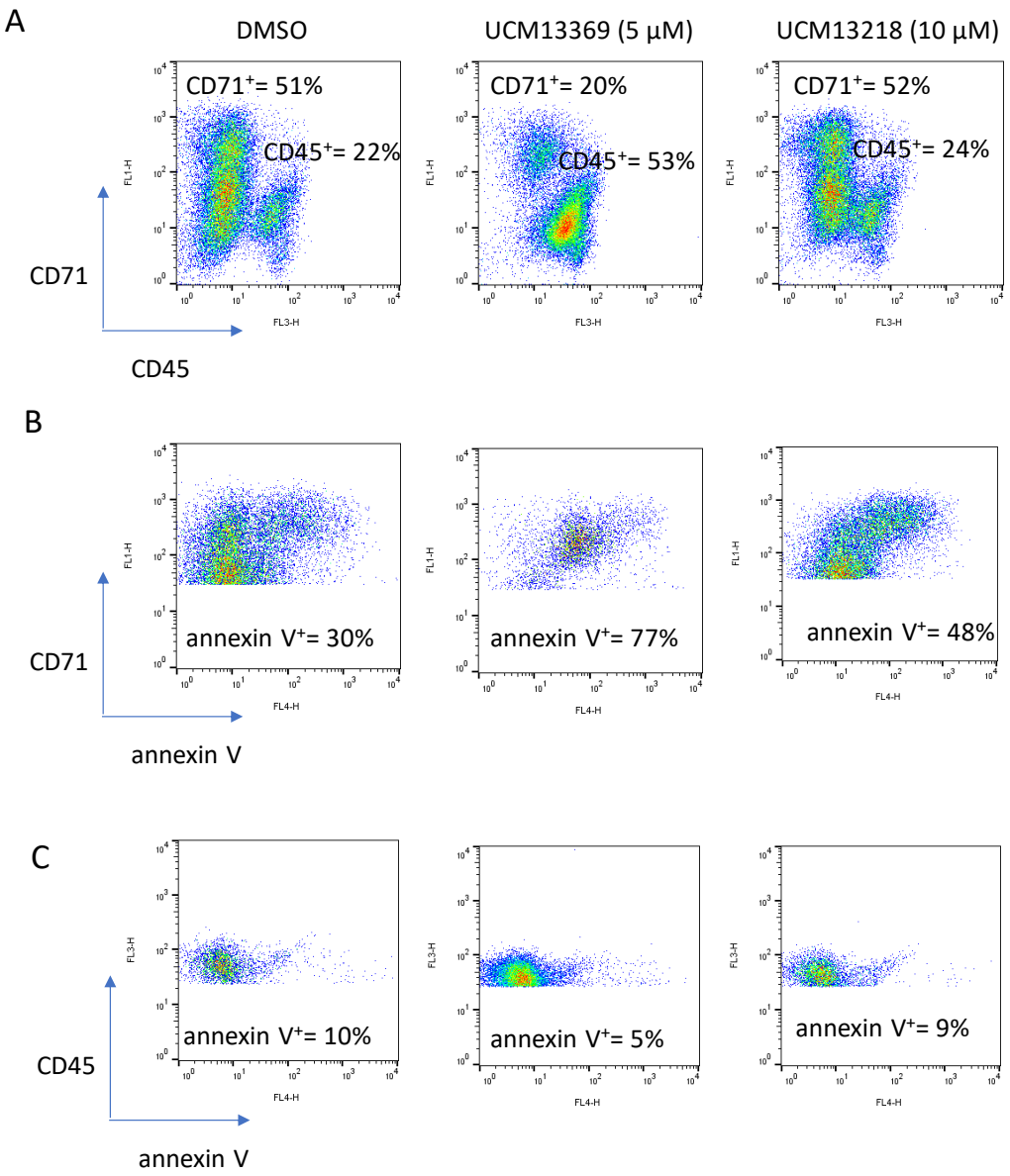


Figure 25. Flow cytometry analysis of $CD71^+$-$CD45^+$(A), $CD71^+$-annexin V^+ (B), and $CD45^+$-annexin V^+ (C) populations after 14 days of treatment of healthy $CD34^+$ cells with UCM13369 (5 µM) and UCM13218 (10 µM)

2.9. Current studies for identified candidates

Mechanism of action: Differential proteomic analysis

Proteomics allows the study of a biological system through the analysis of its proteome, which is the entire set of proteins that is produced or modified by the system. Proteome differs from cell to cell and from time to time. Proteomic analysis based on mass spectrometry and bioinformatics has enabled identification and quantification of an ever-increasing number of proteins. In particular, differential proteomics compares protein profiles in normal, diseased, and drug-treated samples, which is useful to postulate the mechanism of drug action and provides insight for new drug discovery.

iTRAQ (isobaric tags for relative and absolute quantification) is a powerful technology for differential proteomics in complex mixtures. This method utilizes isobaric reagents to label the primary amines of peptides from different samples, allowing to assess quantitative changes of the proteomes under study in a single experiment. iTRAQ reagents usually consist of a reporter group, a balance group, and a peptidic reactive group that reacts with the amino group of the peptides from protein digestions. These reagents have the same total mass, but the mass of the reporter group differs from one reagent to another. Therefore, peptides derivatized with a certain iTRAQ reagent produce specific reporter ions which can be used to quantify the peptides and the proteins from which they originated (Figure 26). [77]

Specifically, samples for iTRAQ analysis are prepared under different treatment conditions (control and treated samples) followed by cell lysis to extract proteins. These proteins are digested with trypsin to the corresponding peptides, and each peptide digest is labelled with a different iTRAQ reagent. All the labelled digests are combined in a single mixture, which is analysed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The fragmentation data are used to identify the labelled peptides and hence the corresponding proteins, whereas the signals of the specific iTRAQ reporter ions allow for quantification of the proteins in each sample (Figure 26).

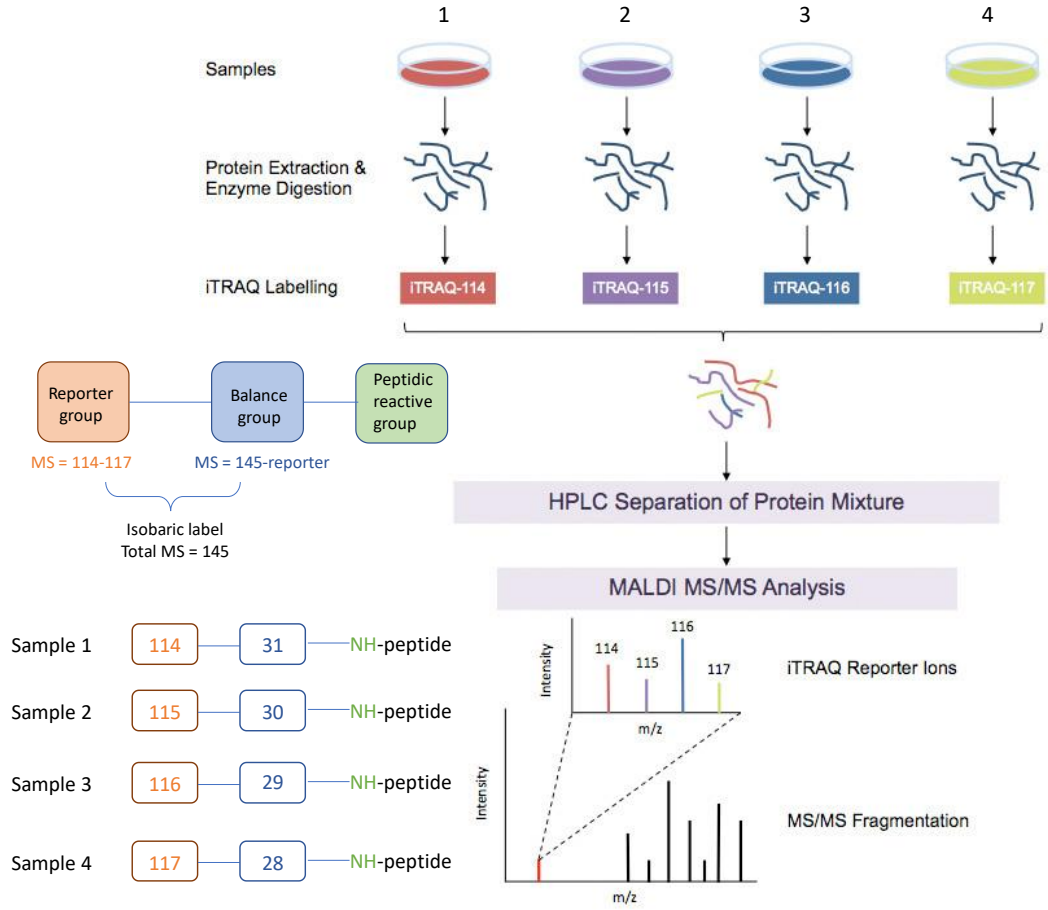


Figure 26. iTRAQ labelling for the identification and quantification of proteins in four different samples (adapted: www.creative-proteomics.com)

In this context, the identification of target protein(s) responsible for the phenotype of compounds UCM13369 and UCM13218 is approached through differential proteomic experiments using iTRAQ. Thus, the proteome of treated $CD34^+$ cells isolated from the patient blood samples is compared with that from non-treated cells, in order to know what proteins are over-expressed or down-expressed after treatment.

Prior to carry out proteomic experiments, the cytotoxicity of tested compound in isolated $CD34^+$ cells was evaluated to ensure a large quantity of protein, using a colorimetric assay based on metabolic reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1). Remaining viable cells are able to reduce WST-1 to orange soluble formazan crystals, which are quantified by measuring their absorbance at 450 nm relative to

the non-treated cells. From the results in Table 8, non-toxic concentrations of 5 µM for UCM13369 and 10 µM for UCM13218, at which the compounds were active in the CFU assay (see Figure 21), were then used to carry out the proteomic experiments. CD34+ cells were incubated in the presence or absence of tested compound for 24 h, and were then lysed. The digestion of proteins, the labelling of corresponding peptides with iTRAQ reagents, and the analysis through LC-MS/MS are in course in collaboration with Dr. Alberto Paradela from Centro Nacional de Biotecnología (CNB) in Madrid.

Table 8. Cytotoxicity of UCM13369 and UCM13218 in $CD34^+$ cells[a]

Compd	Cytotoxicity (%) (@10 µM)
UCM13369	42 ± 4 20 ± 5[b] 2 ± 2[c]
UCM13218	15 ± 4 12 ± 3[b]

[a]Data from two to three independent experiments performed in triplicate; [b]@5 µM; [c]@1 µM.

Therapeutic potential in vivo

In view of the interesting results obtained in the *ex-vivo* AML model, we proposed to study compounds UCM13369 and UCM13218 in an *in-vivo* CSC model.

If one wants to know whether a patient's tumor will respond to a specific therapeutic regimen, one must examine the response of that human tumor, not a mouse tumor, to the therapy. Hence, one of the most widely used models to examine response to therapy is the human tumor xenograft (Figure 27). In this model, human tumor cells are transplanted either under the skin –heterotopic transplantation– or into the organ type in which the tumor originated –orthotopic transplantation–, into immunocompromised mice (athymic nude mice or severely compromised immune deficient –SCID– mice). In particular, xenotransplantation of patient-derived AML cells in nude/SCID mice is the method of choice for evaluating this human hematologic malignancy.[76] Therefore, compounds UCM13369 and UCM13218 are being assessed for their potential therapeutic in leukemia in this human AML xenograft.

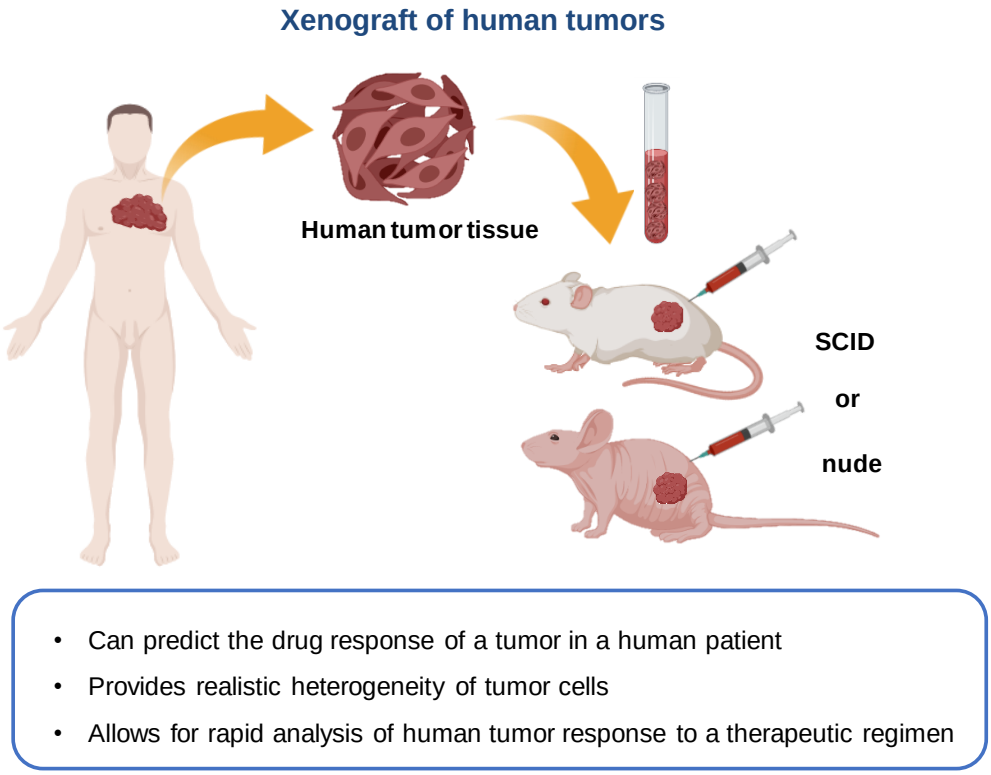


Figure 27. *In-vivo* xenograft model for studying human cancers (adapted: reference 76)

Prior to testing the compounds in this model, the pharmacokinetic properties in healthy mice were studied. After intraperitoneal (ip) administration of a 25 mg/Kg dose of UCM13369, samples of blood were withdrawn at different times from 30 min to 6 h, and the presence of compound was analysed by HPLC-MS. The area of the molecular peak allowed to calculate the concentration of the compound present in mice serum at each time. Using the data from this curve (Figure 28), the pharmacokinetic parameters were obtained for a single-compartment model. From the data in Table 9, UCM13369 exhibits a good pharmacokinetic profile *in vivo* showing a half-life time ($t_{1/2}$) higher than 2 h.

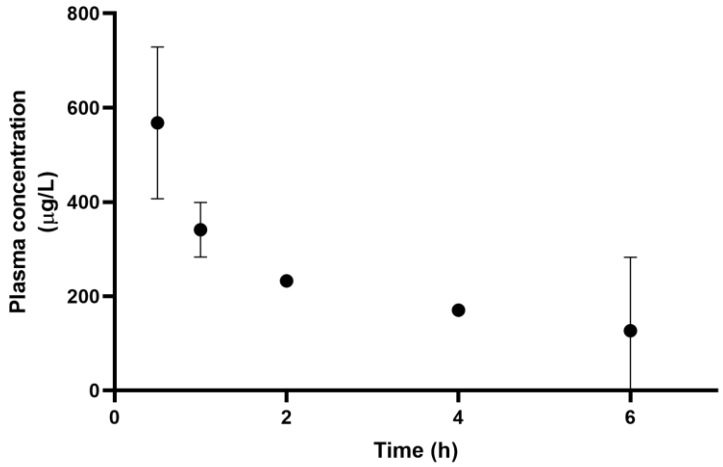


Figure 28. Pharmacokinetic study for UCM13369 (25 mg·Kg^{-1}, ip)

Table 9. Pharmacokinetic parameters obtained for compound UCM13369

Parameter	Value
Dose (D)	25 mg·Kg^{-1}
Initial concentration (C_0)[a]	558 µg·L^{-1}
V_D (= D/C_0)	45 L·Kg^{-1}
k_e[a]	0.34 h^{-1}
$t_{1/2}$ (= ln 2/k_e)	2.01 h
AUC (= C_0/k_e)	1.64 mg·h·L^{-1}
CL (= V_D·k_e)	15 L·Kg^{-1}·h^{-1}

[a] Parameters obtained from lineal regression: ln([UCM13369]) = ln(C_0) - k_e·t.

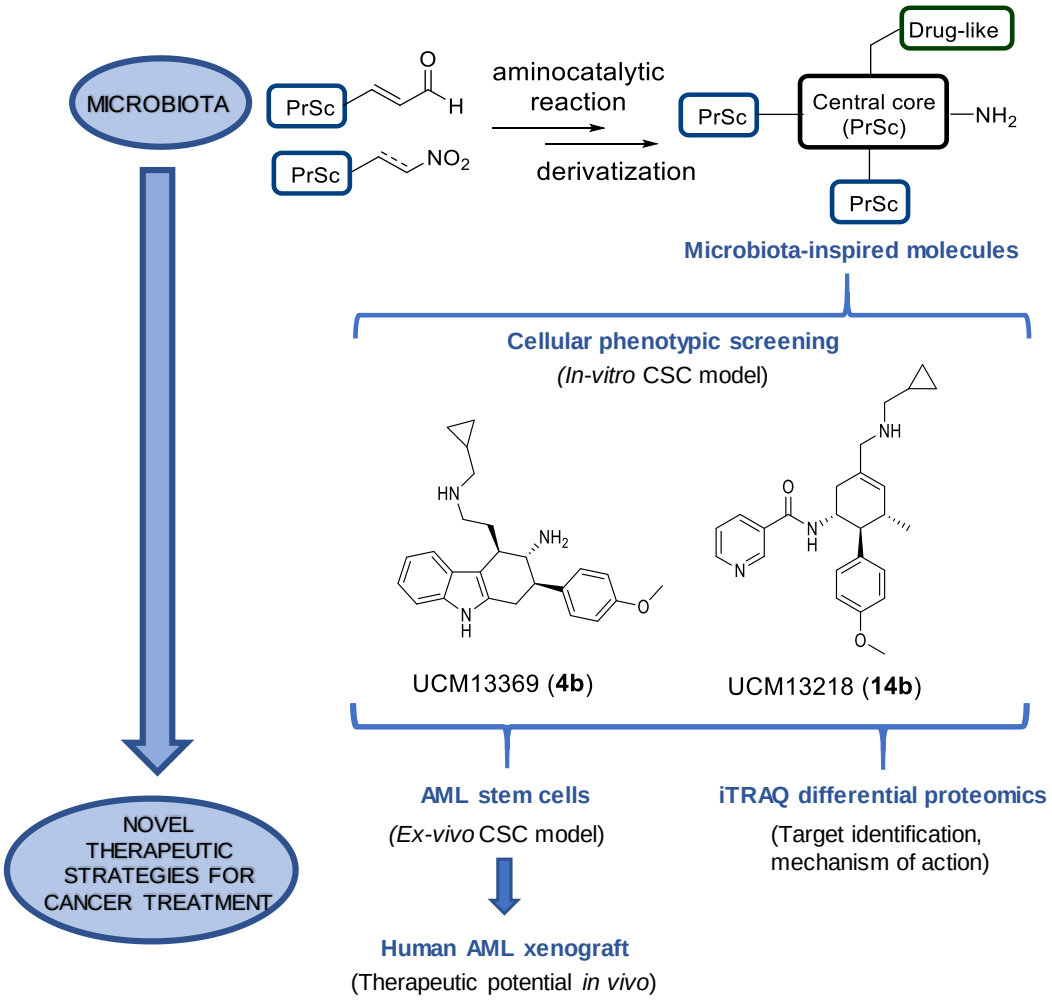
CONCLUSIONS

3. CONCLUSIONS

The present work is focused on the development of new molecules inspired on human microbiota metabolites aimed to target cancer phenotypes, specifically CSC models. New compounds **1**-**9**, **11**-**20**, **46**-**48**, **55**, and **57** were obtained in a diastereo- and enantioselective manner, using asymmetric organocatalytic reactions as a key synthetic step to generate structural diversity based on privileged scaffolds present in microbiota metabolites.

Cellular phenotypic screening assays allowed us to identify three different scaffolds: tetrahydrocarbazole, tetrahydrobenzo[*c*]chromene, and tetrasubstituted cyclohexene present in active compounds able to act on CSCs by promoting their differentiation and/or death. Among them, compounds **4b** (UCM13369) and **14b** (UCM13218) exhibited inhibition of tumorsphere formation by CSCs, and no cytotoxicity in tumor and non-tumor cells.

The therapeutic potential of UCM13369 and UCM13218 was assessed in blood samples of AML patients as an *ex-vivo* CSC model. This assay showed that UCM13218 and UCM13369 are able to induce cell death in AML hematopoietic stem cells without affecting differentiated blood cells, and UCM13369 also promotes myeloid differentiation to leukocytes. Both compounds are currently under study in a human AML xenograft in immunocompromised mice to confirm their *in-vivo* efficacy. In addition, differential proteomic analysis using iTRAQ methodology is in course to identify drug target protein(s) and study the mechanism of action.


MICROBIOTA
PrSc
PrSc
aminocatalytic reaction
derivatization
Drug-like
Central core (PrSc)
PrSc
PrSc
NH2
Microbiota-inspired molecules
Cellular phenotypic screening
(In-vitro CSC model)
UCM13369 (4b)
UCM13218 (14b)
AML stem cells
(Ex-vivo CSC model)
iTRAQ differential proteomics
(Target identification, mechanism of action)
Human AML xenograft
(Therapeutic potential in vivo)
NOVEL THERAPEUTIC STRATEGIES FOR CANCER TREATMENT

EXPERIMENTAL SECTION

4. EXPERIMENTAL SECTION

4.1. Synthesis and characterization

The starting materials, reagents, and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Acros, ABCR, Fluorochem, Scharlab, or Panreac. Dichloromethane (DCM), tetrahydrofuran (THF) and diethyl ether were dried using a Pure Solv^{TM} Micro 100 Liter solvent purification system. Chloroform was dried over P_2O_5 and distilled before using. All reactions were carried out under an argon atmosphere in oven-dried glassware unless otherwise stated. Reactions under MW irradiation were performed in a Biotage Initiator 2.5 reactor.

Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F-254), with detection by UV light (λ = 254 nm), 5% ninhydrin solution in ethanol or 10% phosphomolybdic acid solution in ethanol. Unless otherwise stated, products were purified in a Varian 971-FP system with cartridges of silica gel (Varian, size particle 50 µM).

All compounds were obtained as oils, except for those whose melting points (m.p.) are indicated, which were solids. M.p. (uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 cm^{-1} transmission range; frequencies (ν) are expressed in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance III 700 MHz (^{1}H, 700 MHz) or Bruker DPX 300 MHz (^{1}H, 300 MHz; ^{13}C, 75 MHz) instrument at (rt) at the Universidad Complutense de Madrid (UCM) NMR core facility. Bruker DPX 300 MHz equipment was used unless otherwise stated. Chemical shifts (δ) are expressed in parts per million relative to the residual solvent peak for ^{1}H and ^{13}C nucleus ($CDCl_3$: δ_H = 7.26, δ_C = 77.16; methanol-*d4*: δ_H = 3.31, δ_C = 49.00); coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), app (apparent), and br (broad). 2D NMR experiments –homonuclear

correlation spectroscopy (H,H-COSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC)– of representative compounds were acquired to assign protons and carbons of new structures, and the following abbreviations have been used for the peak assignment: cpr (cyclopropane), ind (indole), py (pyridine), and thz (thiazole). The relative configuration of the compounds was confirmed by 1D ^{1}H-NMR NOE experiments, in which the signal of interest was irradiated with a selective pulse and NOE interactions with this signal were observed.

For all final compounds, purity was determined by HPLC-MS using an Agilent 1200LC-MSD VL instrument, and satisfactory chromatograms confirmed a purity of at least 95% for all tested compounds. LC separation was achieved with a Zorbax SB-C3 column (5 µm, 2.1 mm x 50 mm) or an Eclipse XDB-C18 column (5 µm, 4.6 mm x 150 mm), together with a guard column (5 µm, 4.6 mm x 12.5 mm). The mobile phase consisted of A (95:5 water/ACN) and B (5:95 water/ACN) with 0.1% ammonium hydroxide and 0.1% formic acid as solvent modifiers, and the gradients are indicated in Table 10. MS analysis was performed with an electrospray irradiation source. The capillary voltage was set to 3.0 kV and the fragmentor voltage to 72 or 35 eV. The drying gas temperature was 350 ºC, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. Spectra were acquired in positive or negative ionization mode from 80 to 800 *m/z* and in UV-mode at four different wavelengths (210, 230, 254, and 280 nm).

Table 10. HPLC gradient

t (min)	%B
0	0
2	0
10	50
20	100
25	100
30	0

Optical rotation [α] was measured on an Anton Paar MCP 100 modular circular polarimeter using a sodium lamp (λ = 589 nm) with a 1 dm path length; concentrations (c) are given as g/100 mL. The *ee* was determined by HPLC using a chiral column (Chiralpak® IA or IC, 5 µm, 4.6 mm x 150 mm) in reversed- or normal-phase chromatography. Methods A-F were employed for reversed-phase conditions (Table 11) and methods H-J were used for normal-phase (Table 12). HPLC traces were compared to racemic samples obtained by mixing equal amounts of the enantiopure compounds independently obtained.

Table 11. Reversed-phase chiral HPLC

Method	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Column	IA	IA	IA	IA	IC	IC	IC
Eluent (20 mM NH_4HCO_3 pH 9/ ACN)	40:60	60:40	50:50	40:60	70:30	85:15	30:70
Flow (mL/min)	0.6	0.5	0.8	0.8	0.6	0.5	0.6

Table 12. Normal-phase chiral HPLC

Method	**H**	**I**	**J**
Column	IA	IA	IC
Eluent (hexane/ ^{i}PrOH)	40:60	30:70 (0.1% EDA)[a]	30:70 (0.1% EDA)[a]
Flow (mL/min)	0.6	0.8	0.8

[a] EDA: ethylenediamine

Final compounds **1**-**3**, **5**-**9**, **11**-**20**, **47**, **55**, and **57** were characterized (α, R_f, IR, NMR, HPLC-MS) and subsequently transformed into the corresponding hydrochloride salts. Thus, a commercial solution of 2 M HCl(g) in diethyl ether (3 mL/mmol) was added to a solution of the free base in anhydrous DCM or methanol (6 mL/mmol). The resulting salt was isolated by filtration or evaporation of the solvents, washed with anhydrous diethyl ether and dried under vacuum. The purity of the salts was determined by HPLC-MS and elemental chemical analysis (C, H, N, S), which was carried out using a LECO CHNS-932 instrument at Servicio Interdepartamental de Investigación of the Universidad Autónoma de Madrid, and is within 0.5% of the theoretical values, confirming a purity of at least 95% for all compounds tested.

IUPAC rules have been followed to name all organic compounds, except for (diethoxymethoxy)ethane, *p*-methylbenzenesulfonic acid, *N*,*N*-dimethylpyridin-4-amine, (2*E*)-3-(*p*-methoxyphenyl)prop-2-enal, (*E*)-2-(2-nitrovinyl)phenol, 4-methoxy-2-[(*E*)-2-nitrovinyl]phenol, and oxido-[oxido(dioxo)chromio]oxy-dioxochromiun pyridin-1-ium, whose common names triethyl orthoformate, *p*-toluenesulfonic acid, 4-dimethylaminopyridine (DMAP), *p*-methoxycinnamaldehyde, *trans*-2-hydroxy-*β*-nitrostyrene, *trans*-3-hydroxy-5-methoxy-*β*-nitrostyrene, and pyridinium dichromate (PDC), respectively, have been employed for simplicity.

4.1.1. General synthetic procedures

General procedure A: Boc deprotection. To a solution of the corresponding *N*-Boc-protected amide or amine (1.00 eq) in anhydrous DCM (5 mL/mmol), TFA (10.0 eq) was added and the reaction was stirred at rt until TLC showed complete consumption of starting material. The corresponding free amide or amine was isolated following the appropriate work-up:

- For amide derivatives (work-up A): The mixture was concentrated and the residue was dissolved in EtOAc and washed with a sat. $NaHCO_3$ solution (x2). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the corresponding free amide, which was used in the next step without further purification.

- For amine derivatives (work-up B): The mixture was diluted with DCM and extracted with water (x2). The combined aqueous layers were basified (pH > 10) with a sat. $NaHCO_3$ solution, and extracted with EtOAc (x2). The organic layers were washed with brine, dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford the corresponding free amine, which was used in the next step without further purification.

General procedure B: one-pot reductive amination. To a solution of the corresponding aldehyde (1.00-1.50 eq) in anhydrous methanol (5 mL/mmol) and DCM (2 mL/mmol) (only in those cases where the aldehyde is not soluble), the appropriate amine (1.00-2.00 eq) was added and the reaction mixture was stirred at rt for 2-4 h to form the corresponding imine (confirmed by ^{1}H-NMR analysis of an aliquot). Then, $NaBH_4$ (2.00 eq) was added at 0 ºC and the mixture was allowed to react at rt for 3 h. Then, the reaction was quenched with a sat. $NaHCO_3$ solution. The solvent was evaporated and the residue was suspended in water and extracted with EtOAc (x2). The organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was purified by flash chromatography or used in the next step without further purification.

General procedure C: nitro group reduction with Zn/acetic acid. To a solution of the corresponding nitro derivative (1.00 eq) in a 1:1 mixture of acetic acid and methanol (4 mL/mol), zinc (10.0 eq) was added and the reaction was stirred until complete conversion of starting material (1-3 h). Then, the mixture was filtered, (washing with methanol) and the filtrate was evaporated. The residue was suspended in a sat. $NaHCO_3$ solution and extracted with DCM (x3). The combined

organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography to afford the desired amine.

General procedure D: Pinnick oxidation. To a solution of the corresponding aldehyde (1.00 eq) in *tert*-butanol (5 mL/mmol) and anhydrous THF (1 mL/mmol), 2-methylbut-2-ene (1.20 eq), sodium chlorite (1.20 eq) and a sat. solution of K_2HPO_4 (0.4 mL/mmol) were added successively. The mixture was stirred at 30 ºC overnight. The reaction was then quenched with water, and a sat. $NaHCO_3$ solution was added until pH 9. After stirring the mixture for 30 min, the organic layer was discarded. The aqueous phase was treated with 1 M HCl until pH 2, stirred for 30 min and extracted with EtOAc (x2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum to afford the desired carboxylic acid, which was used in the next step without further purification.

General procedure E: one-pot synthesis of amides via acid chloride. To a solution of the corresponding carboxylic acid (1.00 eq) in anhydrous DCM (15 mL/mmol) at 0 ºC, oxalyl chloride (1.00 eq, 2 M in DCM) and DMF (cat.) were added, and the solution was stirred at rt for 1 h. Then, (cyclopropylmethyl)amine (1.50 eq) was added at 0 ºC, and the reaction was allowed to warm up to rt and stirred until complete conversion of starting material (3-18 h). Next, the mixture was washed with a sat. $NaHCO_3$ solution (x2) and a 1:1 mixture of water/brine (x4). The organic layer was dried over Na_2SO_4 and filtered, and concentrated under vacuum to afford the desired amide, which was used in the next step without further purification.

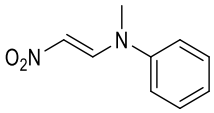
General procedure F: demethylation of methoxy groups. To a solution of the corresponding methoxyaryl derivative (1.00 eq) in anhydrous DCM (10 mL/mmol), BBr_3 (5.00 eq, 1 M in DCM) was added at 0 ºC and the reaction mixture was stirred at this temperature until complete conversion of starting material (1-2 h). Then, water was added and the mixture was stirred for 20 min. Next, the aqueous phase was washed with EtOAc, basified with a sat. $NaHCO_3$ solution until pH 9 and extracted with EtOAc (x2). The combined organic extracts were dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford the corresponding phenol without further purification.

General procedure G: synthesis of amides from nicotinic or valeric acid. A solution of nicotinic or valeric acid (1.00 equiv), HOBt (1.00 eq), EDC (1.10 equiv) and DIPEA (1.10 eq) in anhydrous DCM (5 mL/mmol) was stirred at rt for 40 min.

Then, a solution of the corresponding amine (1.00 eq) in anhydrous DCM (5 mL/mmol) was added at 0 ºC and the reaction was stirred at this temperature for 3 h. Next, the mixture was diluted with EtOAc, and successively washed with water, a 1 M K_2CO_3 solution, and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography to afford the corresponding amide.

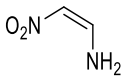
*4.1.2. Synthesis of piperidine scaffold. Final compounds **1**-**3***

***N*-Methyl-*N*-[(*E*)-2-nitroethenyl]aniline, 22.**[68] A mixture of *N*-methylaniline (2.00 mL, 18.7 mmol), nitromethane (3.30 mL, 61.6 mmol), triethyl orthoformate (4.00 mL, 24.3 mmol) and *p*-toluenesulfonic acid monohydrated (87 mg, 0.5 mmol) was heated under reflux for 7 h. Then, the reaction was cooled to rt and concentrated. The residue was purified by recrystallization from toluene/hexane to afford intermediate **22** as a golden solid (1.50 g, 45%). The spectroscopic data were consistent with those previously reported.[78]



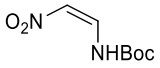
M.p.: 90-93 °C (lit.[78] 91-92 °C). R_f: 0.39 (DCM). ^{1}H-NMR ($CDCl_3$) δ 3.33 (s, 3H, CH_3), 6.85 (d, *J* = 10.8, 1H, $CHNO_2$), 7.17-7.31 (m, 3H, $3CH_{Ar}$), 7.38-7.48 (m, 2H, $2CH_{Ar}$), 8.49 (d, *J* = 10.8, 1H, CHN).

(*Z*)-2-Nitroethen-1-amine, 23. Ammonia (40.80 mL, 0.5 M in 1,4-dioxane) was added to compound **22** (700 mg, 4.08 mmol) and the mixture was allowed to stand at 5 ºC (refrigerator) overnight. Next, the solvent was evaporated under reduced pressure and the residue was purified by glass flash chromatography (DCM to DCM/methanol 9:1) to yield compound **23** as a white solid (150 mg, 43%). The spectroscopic data were consistent with those previously reported.[79]



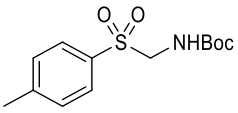
M.p.: 98-100 °C (lit.[79] 101 °C). R_f: 0.54 (DCM/methanol, 9:1). ^{1}H-NMR (methanol-*d4*): δ 6.47 (d, *J* = 5.7, 1H, $CHNO_2$), 6.88 (d, *J* = 10.8, 1H, NH), 6.99-7.09 (m, 1H, $C\underline{H}NH_2$), 8.23 (d, *J* = 10.8, 1H, NH).

***tert*-Butyl [(*Z*)-2-nitroethenyl]carbamate, 24.** A stirred solution of compound **23** (122 mg, 1.34 mmol) in anhydrous DCM (2.2 mL) was cooled at 0 °C and di-*tert*-butyl dicarbonate (363 mg, 1.67 mmol) was added, followed by DMAP (8 mg, 0.07 mmol). The mixture was stirred at rt for 15 min, and then, the reaction was quenched with water and extracted with DCM (x2). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford compound **24** as an oil (261 mg, quantitative), which was used in the next step without further purification.



R_f: 0.53 (hexane/EtOAc, 8:2). IR (ATR): ν 3366 (NH), 1745 (C=O), 1645 (C=C), 1149 (COC). ^{1}H-NMR ($CDCl_3$): δ 1.53 (s, 9H, $3CH_3$), 6.56 (d, *J* = 6.8, 1H, $CHNO_2$), 7.36 (dd, *J* = 12.6, 6.8, 1H, C<u>H</u>NH), 9.69 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 28.1 (CH_3), 84.2 (C), 117.2 ($CHNO_2$), 134.4 (<u>C</u>HNH), CO not observed.

***tert*-Butyl [(4-methylbenzene-1-sulfonyl)methyl]carbamate, 25.**[80] A suspension of t*ert*-butyl carbamate (2.00 g, 17.08 mmol) and *p*-methylbenzenesulfinic acid sodium salt (5.20 g, 29.00 mmol) in a 1:2 mixture methanol/water (15 mL), paraformaldehyde (3.80 mL, 35% aqueous solution) and formic acid (3.0 mL, 6.80 mmol) were added. The reaction mixture was refluxed for 1.5 h, and then it was allowed to reach rt and stirred overnight. Then, the resulting precipitated solid was filtered, washed with water and dried under vacuum to obtain intermediate **25** as a white solid (3.00 g, 60%). The spectroscopic data were consistent with those previously reported.[80]



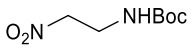
M.p.: 117-118 °C (lit.[80] 119 °C). R_f: 0.40 (hexane/EtOAc, 6:4). ^{1}H-NMR ($CDCl_3$): δ 1.28 (s, 9H, $3CH_3$), 2.44 (s, 3H, CH_3), 4.50 (d, *J* = 7.0, 2H, NHC<u>H</u>$_2$), 5.33 (br s, 1H, NH), 7.36 (d, *J* = 8.0, 2H, $2CH_{Ar}$), 7.80 (d, *J* = 8.0, 2H, $2CH_{Ar}$).

***tert*-Butyl (2-nitroethyl)carbamate, 21**

Method A:[67] To a solution of compound **24** (266 mg, 1.41 mmol) in anhydrous methanol (4.5 mL) at 0 ºC, $NaBH_4$ (107 mg, 2.82 mmol) was added portionwise. The mixture was allowed to warm up to rt and stirred for 2 h. Then, the reaction

was quenched by addition of a sat. NH_4Cl solution and the mixture was extracted with EtOAc (x2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash chromatography (hexane to hexane/EtOAc 8:2) to give title product **21** as an oil (139 mg, 52%). The spectroscopic data were consistent with those previously reported.[67]

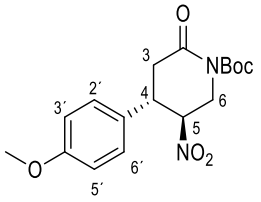
Method B: To a suspension of K_2CO_3 (306 mg, 2.2 mmol) in anhydrous THF (9.5 mL), nitromethane (1.0 mL, 18.4 mmol) was added and the mixture was stirred at rt for 30 min. Then, compound **25** (500 mg, 1.80 mmol) was added and the mixture was heated at 120 ºC under MW for 30 min. After that time, the reaction was filtered and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (hexane to hexane/EtOAc 8:2) to afford title compound **21** as an oil (165 mg, 50%). The spectroscopic data were consistent with those previously reported.[67]



R_f: 0.22 (hexane/EtOAc, 8:2). ^{1}H-NMR ($CDCl_3$): δ 1.45 (s, 9H, $3CH_3$), 3.70 (dd, *J* = 10.5, 6.0, 2H, $C\underline{H}_2NH$), 4.51 (app t, *J* = 5.3, 2H, CH_2NO_2), 5.01 (br s, 1H, NH).

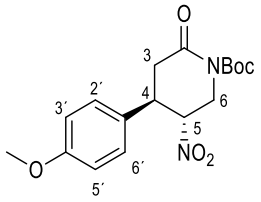
***tert*-Butyl 4-(4-methoxyphenyl)-5-nitro-2-oxopiperidine-1-carboxylate, 26.** To a suspension of (*S*)- or (*R*)- 2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.10 eq), **21** (1.50 eq) and benzoic acid (0.20 eq) in anhydrous DCM (1.5 mL/mmol) at 0 ºC, *p*-methoxycinnamaldehyde (1.00 eq) was added. The reaction mixture was stirred at 0 ºC for 1 h and then it was allowed to warm up to rt and stirred for 4 h. Next, the solution was diluted with anhydrous DCM (10 mL/mmol), 4 Å molecular sieves (700 mg) and PDC (3.00 eq) were added, and the reaction was stirred overnight. Then, the mixture was filtered through silica gel, and the filtrate was concentrated and purified by flash chromatography (hexane to hexane/EtOAc 85:15) to afford title compound (4*S*,5*S*)- or (4*R*,5*R*)-**26**.

(4*S*,5*S*)-**26** (**26a**). Following the previous procedure, compound **26a** (dr = 9:1) was obtained from *p*-methoxycinnamaldehyde (212 mg, 1.31 mmol), using the *S* enantiomer of the catalyst (40 mg, 0.13 mmol), as an oil (205 mg, 45%).



R_f: 0.37 (hexane/EtOAc 7:3). IR (ATR): ν 1775 (C=O), 1720 (C=O), 1252 (COC). ^{1}H-NMR ($CDCl_3$, major diastereoisomer): δ 1.55 (s, 9H, $3CH_3$), 2.76 (dd, *J* = 16.7, 10.6, 1H, H_3), 2.89 (dd, *J* = 16.7, 5.8, 1H, H_3), 3.79-3.82 (m, 1H, H_4), 3.80 (s, 3H, OCH_3), 4.00 (dd, *J* = 14.5, 4.9, 1H, H_6), 4.56 (dd, *J* = 14.5, 5.1, 1H, H_6), 4.89 (dt, *J* = 7.5, 5.0, 1H, H_5), 6.90 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.13 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$, major diastereoisomer): δ 28.1 ($3CH_3$), 39.6 (C_3), 41.3 (C_4), 46.1 (C_6), 55.7 (OCH_3), 84.5 ($\underline{C}(CH_3)_3$), 86.0 (C_5), 114.9 ($C_{3'}$, $C_{5'}$), 128.1 ($C_{2'}$, $C_{6'}$), 130.6 ($C_{1'}$), 151.5 (NCOO), 159.6 ($C_{4'}$), 168.4 (C_2). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.00 ppm (dd, H_6) yielded NOE on 3.77-3.86 (m, H_4); and irradiation of the signal at δ 4.56 ppm (dd, H_6) yielded NOE on 4.89 (dt, H_5), and 7.13 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 17.34. MS (ESI, *m/z*, %): 351.2 ([M+H]$^+$, 100).

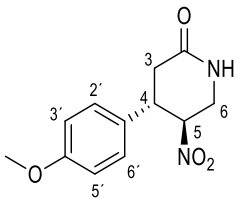
(4*R*,5*R*)-**26** (**26b**). Following the previous procedure, compound **26b** (dr = 9:1) was obtained from 4-methoxycinnamaldehyde (240 mg, 1.48 mmol), using the *R* enantiomer of the catalyst (46 mg, 0.15 mmol), as an oil (217 mg, 58%).



Spectroscopic data were in agreement with those described for enantiomer **26a**.

4-(4-Methoxyphenyl)-5-nitropiperidin-2-one, 27

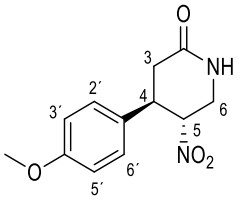
(4*S*,5*S*)-**27** (**27a**). Following general procedure A (work-up A), compound **27a** (dr = 85:15) was obtained from **26a** (112 mg, 0.32 mmol) as an oil (68 mg, 85%).



R_f: 0.17 (EtOAc). IR (ATR): ν 3226 (NH), 1672 (C=O), 1522 (NO_2), 1252 (COC). ^{1}H-NMR ($CDCl_3$, major diastereoisomer): δ 2.67 (dd, *J* = 18.1, 8.5, 1H, H_3), 2.82 (dd, *J* = 18.1, 6.3, 1H, H_3), 3.72 (ddd, *J* = 12.9, 4.9, 2.7, 1H, H_6), 3.80 (s, 3H, CH_3), 3.82-3.92 (m, 1H, H_4), 3.95 (ddd, *J* = 12.8, 6.9, 1.9, 1H, H_6), 4.87 (ddd, *J* = 8.1, 6.9, 4.9, 1H, H_5), 6.56 (s, 1H, NH), 6.89 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.16 (d, *J* =

8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$, major diastereoisomer): δ 35.3 (C_3), 41.5 (C_4), 43.1 (C_6), 55.5 (CH_3), 84.6 (C_5), 114.9 ($C_{3'}$, $C_{5'}$), 128.3 ($C_{2'}$, $C_{6'}$), 130.0 ($C_{1'}$), 159.7 ($C_{4'}$), 170.2 (C_2). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.87 ppm (ddd, H_5) yielded NOE on 7.16 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 14.25. MS (ESI, *m/z*, %): 251.1 ([M+H]$^+$, 100).

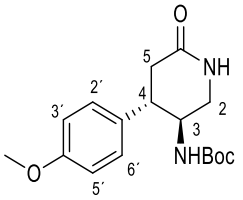
(4*R*,5*R*)-**27** (**27b**). Following general procedure A (work-up A), compound **27b** (dr = 85:15) was obtained from **26b** (65 mg, 0.19 mmol) as an oil (43 mg, 93%).



Spectroscopic data were in agreement with those described for enantiomer **27a**.

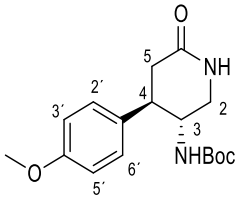
***tert*-Butyl [4-(4-methoxyphenyl)-6-oxopiperidin-3-yl]carbamate, 28.** To a solution of enantiomer **27a** or **27b** (1.00 eq) in anhydrous methanol (7 mL/mmol) at 0 ºC, nickel chloride hexahydrate (0.05 eq) was added and the reaction was stirred at this temperature for 5 min. $NaBH_4$ (4.00 eq) was added portionwise over 30 min and the reaction was stirred at 0 ºC for 30 min. Then, di-*tert*-butyl dicarbonate (1.20 eq) was added and the reaction was allowed to warm up to rt and stirred overnight. Next, the reaction was quenched with a sat. NH_4Cl solution, the mixture was filtered through celite, and the filtrate was evaporated. The residue was dissolved in EtOAc and washed with water, a sat. $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford title compound (3*S*,4*S*)- or (3*R*,4*R*)-**28**, which was used in the next step without further purification.

(3*S*,4*S*)-**28** (**28a**). Following the previous procedure, compound **28a** (dr = 85:15) was obtained from **27a** (102 mg, 0.27 mmol) as an oil (130 mg, quantitative).



R_f: 0.14 (EtOAc). IR (ATR): ν 3292 (NH), 1696 (C=O), 1656 (C=O), 1212 (COC). ^{1}H-NMR ($CDCl_3$, major diastereoisomer): δ 1.36 (s, 9H, $3CH_3$), 2.59 (dd, *J* = 18.0, 9.1, 1H, H_5), 2.74 (dd, *J* = 18.1, 5.9, 1H, H_5), 3.07-3.17 (m, 2H, H_2, H_4), 3.58-3.63 (m, 1H, H_2), 3.80 (s, 3H, OCH_3), 4.02 (br s, 1H, H_3), 4.56 (d, *J* = 7.2, 1H, NHBoc), 6.14 (br s, 1H, NH), 6.88 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.14 (d, *J* = 8.5, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$, major diastereoisomer): δ 28.4 ($3CH_3$), 37.3 (C_5), 42.7 (C_4), 45.8 (C_2), 55.4 (OCH_3), 80.2 ($C(CH_3)_3$), 114.5 ($C_{3'}$, $C_{5'}$), 128.5 ($C_{2'}$, $C_{6'}$), 132.3 ($C_{1'}$), 155.4 (NHCOO), 159.0 ($C_{4'}$), C_3 and C_6 not observed. HPLC (t_R, min): 14.95. MS (ESI, *m/z*, %): 265.1 ([M-O$(CH_3)_3$+NH_4]$^+$, 100).

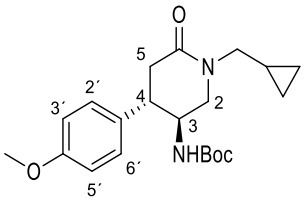
(3*R*,4*R*)-**28** (**28b**). Following the previous procedure, compound **28b** (dr = 85:15) was obtained from **27b** (43 mg, 0.17 mmol) as an oil (54 mg, quantitative).



Spectroscopic data were in agreement with those described for enantiomer **28a**.

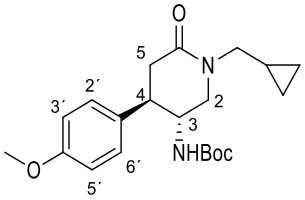
***tert*-Butyl [1-(cyclopropylmethyl)4-(4-methoxyphenyl)-6-oxopiperidin-3-yl]carbamate, 29.** To a solution of enantiomer **28a** or **28b** (1.00 eq) in anhydrous DMF (8 mL/mmol) at 0 ºC, NaH (1.50 eq) was added and the reaction was stirred at this temperature for 1 h. Then, (bromomethyl)cyclopropane (2.00 eq) and NaI (2.00 eq) were added and the mixture was stirred at rt overnight. Then, the mixture was diluted with EtOAc, and washed with water (x2) and a 1:1 mixture of water/brine (x3). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (hexane to hexane/EtOAc 1:1) to yield title compound (3*S*,4*S*)- or (3*R*,4*R*)-**29**, as a single diastereoisomer.

(3*S*,4*S*)-**29** (**29a**). Following the previous procedure, compound **29a** was obtained from **28a** (160 mg, 0.50 mmol) as an oil (78 mg, 42%).



R_f: 0.28 (hexane/EtOAc 4:6). $[\alpha]^D_{20}$ = -10.8 (c = 0.52, $CHCl_3$). IR (ATR): ν 1709 (C=O), 1630 (C=O), 1252 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.20-0.31 (m, 2H, CH_{2cpr}), 0.46-0.57 (m, 2H, CH_{2cpr}), 0.93-1.06 (m, 1H, CH_{cpr}), 1.36 (s, 9H, $3CH_3$), 2.60 (dd, *J* = 17.9, 9.5, 1H, H_5), 2.76 (dd, *J* = 17.9, 5.9, 1H, H_5), 3.06 (td, *J* = 9.3, 6.0, 1H, H_4), 3.17-3.23 (m, 2H, H_2, ½NCH_2), 3.37 (dd, *J* = 13.9, 6.9, 1H, ½NCH_2), 3.67 (dd, *J* = 12.2, 4.9, 1H, H_2), 3.79 (s, 3H, OCH_3), 4.05 (br s, 1H, H_3), 4.49 (br s, NH), 6.87 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.12 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.5 (CH_{2cpr}), 3.8 (CH_{2cpr}), 9.2 (CH_{cpr}), 28.4 ($3CH_3$), 38.1 (C_5), 43.5 (C_4), 51.25, 51.28 (C_2, NCH_2), 55.4 (OCH_3), 80.1 ($\underline{C}(CH_3)_3$), 114.5 ($C_{3'}$, $C_{5'}$), 128.5 ($C_{2'}$, $C_{6'}$), 132.4 ($C_{1'}$), 155.4 (NHCOO), 159.0 ($C_{4'}$), 168.4 (C_6), C_3 not observed. 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.05 ppm (br s, H_3) yielded NOE on 7.12 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 18.50. MS (ESI, *m/z*, %): 375.2 ($[M+H]^+$, 100).

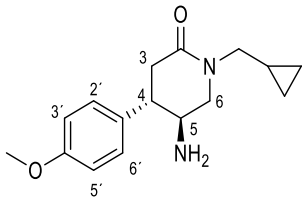
(3*R*,4*R*)-**29** (**29b**). Following the previous procedure, compound **29b** was obtained from **28b** (120 mg, 0.37 mmol) as an oil (50 mg, 36%).



$[\alpha]^D_{20}$= +12.2 (c = 0.44, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **29a**.

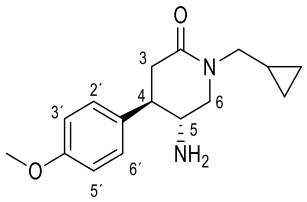
5-Amino-1-(cyclopropylmethyl)-4-(4-methoxyphenyl)piperidin-2-one, 1.

(4*S*,5*S*)-**1** (**1a**). Following general procedure A (work-up B), compound **1a** was obtained from **29a** (52 mg, 0.16 mmol) as an oil (28 mg, 87%, 96% *ee*).



R_f: 0.28 (DCM/methanol 8:2). $[\alpha]^{D}_{20}$ = -9.1 (c = 0.11, $CHCl_3$). Chiral HPLC (method A, t_R, min): 5.35. IR (ATR): ν 3416 (NH), 1633 (C=O), 1252 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.24-0.29 (m, 2H, CH_{2cpr}), 0.50-0.56 (m, 2H, CH_{2cpr}), 0.94-1.06 (m, 1H, CH_{cpr}), 2.55 (dd, *J* = 17.5, 11.4, 1H, H_3), 2.74 (dd, *J* = 17.4, 5.4, 1H, H_3), 2.75-2.86 (m, 1H, H_4), 3.18-3.34 (m, 2H, H_5, H_6), 3.21 (dd, *J* = 14.0, 7.2, 1H, ½NCH_2), 3.41 (dd, *J* = 13.9, 7.0, 1H, ½NCH_2), 3.54-3.60 (m, 1H, H_6), 3.80 (s, 3H, CH_3), 6.90 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.14 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.4 (CH_{2cpr}), 3.7 (CH_{2cpr}), 9.2 (CH_{cpr}), 38.8 (C_3), 47.2 (C_4), 51.3 (NCH_2), 51.4 (C_5), 54.0 (C_6), 55.8 (CH_3), 114.6 ($C_{3'}$, $C_{5'}$), 128.6 ($C_{2'}$, $C_{6'}$), 132.7 ($C_{1'}$), 159.0 ($C_{4'}$), 168.3 (C_2). HPLC (t_R, min): 3.70. MS (ESI, *m/z*, %): 275.2 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{16}H_{22}N_2O_2 \cdot HCl$: %C 61.83, %H 7.46, %N 9.01; experimental: %C 60.71, %H 7.16, %N 8.89.

(4*R*,5*R*)-**1** (**1b**). Following the general procedure A (work-up B), compound **1b** was obtained from **29b** (39 mg, 0.09 mmol) as an oil (21 mg, 73%, 96% *ee*).

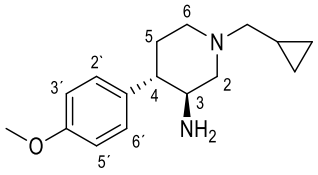


$[\alpha]^{D}_{20}$= +8.50 (c = 0.11, $CHCl_3$). Chiral HPLC (method A, t_R, min): 7.14. Elemental analysis calculated for $C_{16}H_{22}N_2O_2 \cdot HCl$: %C 61.83, %H 7.46, %N 9.01; experimental: %C 61.05, %H 7.26, %N 8.72. Spectroscopic data were in agreement with those described for enantiomer **1a**.

1-(Cyclopropyl)-4-(4-methoxyphenyl)piperidin-3-amine, 2. To a solution of enantiomer **1a** or **1b** (1.00 eq) in anhydrous THF (2 mL/mmol), $LiAlH_4$ (4.00 eq) was added at 0 ºC, and the reaction was warmed up to rt and stirred overnight. Then, a sat. NaOH solution and a sat. Rochelle salt solution were successively added. The mixture was stirred for 20 min, and extracted with EtOAc (x2). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and

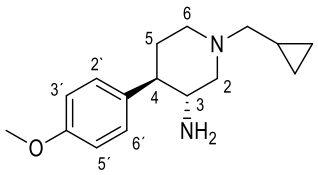
the solvent was evaporated. The residue was purified by flash chromatography (EtOAc to EtOAc/methanol 8:2) to afford (3*S*,4*S*)- or (3*R*,4*R*)-**2**.

(3*S*,4*S*)-**2** (**2a**)**.** Following the previous procedure, compound **2a** was obtained from **1a** (37 mg, 0.14 mmol) as an oil (27 mg, 77%, 96% *ee*).



R_f: 0.28 (EtOAc/methanol 8:2). $[\alpha]^D_{20}$ = -20.8 (c = 1.00, $CHCl_3$). Chiral HPLC (method A, t_R, min): 7.55. IR (ATR): ν 2925 (NH), 1250 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.13-0.18 (m, 2H, CH_{2cpr}), 0.53-0.59 (m, 2H, CH_{2cpr}), 0.89-1.02 (m, 1H, CH_{cpr}), 1.78-1.95 (m, 3H, H_2, $2H_5$), 2.07-2.22 (m, 2H, H_4, H_6), 2.32-2.44 (m, 2H, NCH_2), 3.10 (td, *J* = 10.2, 4.0, 1H, H_3), 3.19 (app d, *J* = 11.6, 1H, H_6), 3.34 (ddd, *J* = 11.0, 4.0, 1.1, 1H, H_2), 3.79 (s, 3H, CH_3), 6.87 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.16 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 4.1 (CH_{2cpr}), 4.3 (CH_{2cpr}), 8.2 (CH_{cpr}), 33.0 (C_5), 51.3 (C_4), 53.0 (C_3), 54.1 (C_6), 55.4 (CH_3), 61.6 (C_2), 63.8 (NCH_2), 114.3 ($C_{3'}$, $C_{5'}$), 128.9 ($C_{2'}$, $C_{6'}$), 135.3 ($C_{1'}$), 158.6 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 7.16 ppm (d, $H_{2'}$, $H_{6'}$) yielded NOE on 3.12 (td, H_3). HPLC (t_R, min): 2.90. MS (ESI, *m/z*, %): 261.2 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{16}H_{24}N_2O \cdot 2HCl \cdot H_2O$: %C 54.70, %H 8.03, %N 7.97; experimental: %C 54.19, %H 7.84, %N 7.67.

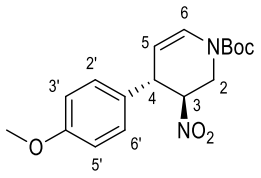
(3*R*,4*R*)-**2** (**2b**). Following the previous procedure, compound **2b** was obtained from **1b** (35 mg, 0.16 mmol) as an oil (28 mg, 85%, 96% *ee*).



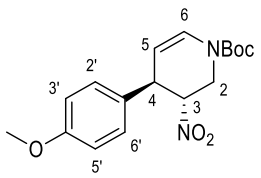
$[\alpha]^D_{20}$= +22.9 (c = 1.00, $CHCl_3$). Chiral HPLC (method A, t_R, min): 9.69. Elemental analysis calculated for $C_{16}H_{24}N_2O \cdot 2HCl \cdot H_2O$: %C 54.70, %H 8.03, %N 7.97; experimental: %C 53.88, %H 7.64, %N 7.77. Spectroscopic data were in agreement with those described for enantiomer **2a**.

***tert*-Butyl 4-(4-methoxyphenyl)-3-nitro-3,4-dihydropyridine-1(2*H*)-carboxylate, 30.** To a suspension of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.10 eq), **21** (1.50 eq) and benzoic acid (0.20 eq) in anhydrous DCM (1.5 mL/mmol) at 0 ºC, 4-methoxycinnamaldehyde (1.00 eq) was added. The reaction mixture was stirred at 0 ºC for 1 h and it was allowed to warm up to rt and stirred for 4 h. Then, the reaction was diluted with anhydrous DCM (10 mL/mmol), TFA (3.00 eq) was added and the reaction was stirred at rt for 2 h. The reaction was quenched with a sat. $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography using hexane to afford (3*S*,4*S*)- or (3*R*,4*R*)-**30**.

(3*S*,4*S*)-**30** (**30a**). Following the previous procedure, compound **30a** (dr = 8:2) was obtained from 4-methoxycinnamaldehyde (150 mg, 0.92 mmol), using the *S* enantiomer of the catalyst (30 mg, 0.09 mmol), as an oil (270 mg, 87%).



R_f: 0.60 (hexane/EtOAc 8:2). IR (ATR): ν 1709 (C=O), 1550 (NO_2), 1253 (COC). ^{1}H-NMR ($CDCl_3$, major diastereoisomer): δ 1.52 (s, 9H, $3CH_3$), 3.79 (s, 3H, OCH_3), 3.89-4.17 (m, 3H, $2H_2$, H_4), 4.61 (br s, H_3), 4.83-4.95 (m, H_5), 6.83 and 7.08 (d, *J* = 8.9, and d, *J* = 8.7, 1H, H_6, amide rotamers), 6.87 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.15 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$, major diastereoisomer): δ 28.3 ($3CH_3$), 41.7 (C_2, C_4), 55.34 (OCH_3), 82.4 ($\underline{C}(CH_3)_3$), 85.3 (C_3), 104.6 (C_5), 128.2 and 130.34 (C_6 amide rotamers), 114.5 ($C_{3'}$, $C_{5'}$), 129.2 ($C_{2'}$, $C_{6'}$), 131.8 ($C_{1'}$), 149.4 (NCOO), 159.7 ($C_{4'}$). HPLC (t_R, min): 23.6. MS (ESI, *m/z*, %): 235.1 ([M-Boc+2H]$^+$, 100).

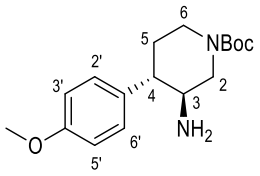


(3*R*,4*R*)-**30** (**30b**). Following the previous procedure, compound **30b** (dr = 8:2) was obtained from 4-methoxycinnamaldehyde (130 mg, 0.80 mmol), using the *R*

enantiomer of the catalyst (26 mg, 0.08 mmol), as an oil (226 mg, 84%). Spectroscopic data were in agreement with those described for enantiomer **30a**.

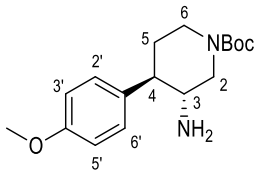
***tert*-Butyl 3-amino-4-(4-methoxyphenyl)piperidine-1-carboxylate, 31.** To a solution of enantiomer **30a** or **30b** (1.00 eq) in anhydrous methanol (10 mL/mmol) at 0 ºC, nickel chloride hexahydrate (2.00 eq) and $NaBH_4$ (10.00 eq) were added portionwise. The resulting black suspension was stirred at this temperature for 5 min and then warmed to rt and stirred for 3 h. An extra portion of $NaBH_4$ (5.00 eq) was added and stirring continued at rt overnight. Then, the reaction was quenched with a sat. NH_4Cl solution, diluted with water and extracted with DCM (x3). The combined organic layers were washed with brine, dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (DCM to DCM/ethanol 95:5) to yield title compound (3*S*,4*S*)- or (3*R*,4*R*)-**31**, as a single diasteroisomer.

(3*S*,4*S*)-**31** (**31a**). Following the previous procedure, compound **31a** was obtained from **30a** (100 mg, 0.30 mmol) as an oil (55 mg, 60%).



R_f: 0.44 (DCM/ethanol 9:1). $[\alpha]^D_{20}$ = +0.62 (c = 0.80, $CHCl_3$). IR (ATR): ν 3368 (NH), 1690 (C=O), 1245 (COC). ^{1}H-NMR ($CDCl_3$): δ 1.48 (s, 9H, $3CH_3$), 1.59-1.77 (m, 2H, $2H_5$), 2.29 (td, *J* = 11.2, 4.2, 1H, H_4), 2.50 (t, *J* = 11.6, 1H, H_2), 2.72-2.87 (m, 2H, H_3, H_6), 3.79 (s, 3H, OCH_3), 4.09-4.29 (m, 2H, H_2, H_6), 6.87 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.14 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 28.6 ($3CH_3$), 33.2 (C_5), 44.2 (br s, C_6), 51.1 (br s, C_2, C_4), 52.9 (C_3), 55.4 (OCH_3), 79.8 ($\underline{C}(CH_3)_3$), 114.3 ($C_{3'}$, $C_{5'}$), 128.8 ($C_{2'}$, $C_{6'}$), 134.7 ($C_{1'}$), 154.8 (NCOO), 158.7 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 7.14 ppm (d, $H_{2'}$, $H_{6'}$) yielded NOE on 2.72-2.87 (m, H_3). HPLC (t_R, min): 13.1. MS (ESI, *m/z*, %): 307.2 ($[M+H]^+$, 100).

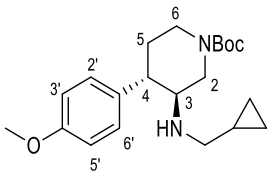
(3*R*,4*R*)-**31** (**31b**). Following the previous procedure, compound **31b** was obtained from **30b** (310 mg, 0.93 mmol) as an oil (130 mg, 53%).



$[\alpha]^{D}_{20}$= -1.08 (c = 1.02, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **31a**.

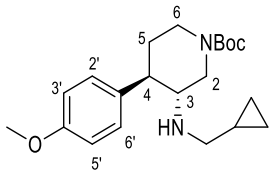
***tert*-Butyl 3-[(cyclopropylmethyl)amino]-4-(4-methoxyphenyl)piperidine-1-carboxylate, 32.**

(3*S*,4*S*)-**32** (**32a**). Following general procedure B, compound **32a** was obtained from **31a** (33 mg, 0.11 mmol) and cyclopropanecarbaldehyde (12 µL, 0.16 mmol) as an oil (25 mg, 63%). Chromatography: hexane to hexane/EtOAc 1:1.



R_f: 0.25 (hexane/EtOAc 4:6). $[\alpha]^{D}_{20}$ = -11.5 (c = 0.90, $CHCl_3$). IR (ATR): ν 1691 (C=O), 1241 (COC). ^{1}H-NMR ($CDCl_3$): δ -0.17-(-0.01) (m, 2H, CH_{2cpr}), 0.28-0.38 (m, 2H, CH_{2cpr}), 0.66-0.78 (m, 1H, CH_{cpr}), 1.48 (s, 9H, $3CH_3$), 1.63-1.76 (m, 2H, $2H_5$), 2.10-2.16 (m, 1H, ½NHC$\underline{H}_2$), 2.39-2.50 (m, 2H, H_4, H_2), 2.55 (dd, *J* = 12.2, 6.5, 1H, ½NHC$\underline{H}_2$), 2.65 (td, *J* = 10.4, 4.1, 1H, H_3), 2.79 (app t, *J* = 11.6, 1H, H_6), 3.79 (s, 3H, OCH_3), 4.15 (app br s, 1H, H_6), 4.43 (app br s, 1H, H_2), 6.86 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.14 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.2 (CH_{2cpr}), 3.6 (CH_{2cpr}), 11.3 (CH_{cpr}), 28.6 ($3CH_3$), 33.8 (C_5), 44.4 (br s, C_6), 48.7 (C_4), 49.1 (br s, C_2), 52.8 ($NHCH_2$), 55.4 (OCH_3), 59.0 (C_3), 79.7 ($\underline{C}(CH_3)_3$), 114.3 ($C_{3'}$, $C_{5'}$), 128.7 ($C_{2'}$, $C_{6'}$), 134.4 ($C_{1'}$), 154.8 (NCOO), 158.7 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 7.14 ppm (d, $H_{2'}$, $H_{6'}$) yielded NOE on 2.65 (td, H_3). HPLC (t_R, min): 14.6. MS (ESI, *m/z*, %): 361.2 ($[M+H]^+$, 100).

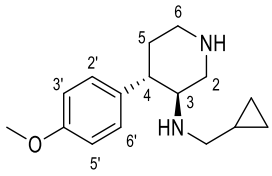
(3*R*,4*R*)-**32** (**32b**). Following general procedure B, compound **32b** was obtained from **31b** (124 mg, 0.40 mmol) and cyclopropanecarbaldehyde (50 µL, 0.61 mmol) as an oil (93 mg, 64%). Chromatography: hexane to hexane/EtOAc 1:1.



$[\alpha]^{D}_{20}$= +12.2 (c = 1.03, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **32a**.

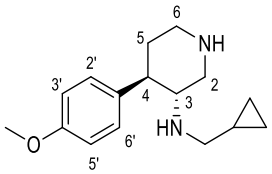
***N*-(Cyclopropylmethyl)-4-(4-methoxyphenyl)piperidin-3-amine, 3**

(3*S*,4*S*)-**3** (**3a**). Following general procedure A (work-up B), compound **3a** was obtained from **32a** (35 mg, 0.10 mmol) as an oil (21 mg, 80%, 96% *ee*).



R_f: 0.20 (DCM/ethanol 9:1). $[\alpha]^{D}_{20}$ = -24.6 (c = 1.00, $CHCl_3$). Chiral HPLC (method G, t_R, min): 8.45. IR (ATR): ν 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ -0.21-(-0.04) (m, 2H, CH_{2cpr}), 0.27-0.37 (m, 2H, CH_{2cpr}), 0.69-0.79 (m, 1H, CH_{cpr}), 1.68-1.81 (m, 2H, $2H_5$), 2.10 (dd, *J* = 12.0, 7.4, 1H, ½NHC$\underline{H}_2$), 2.25 (br s, 2H, 2NH), 2.40-2.54 (m, 3H, H_2, H_4, ½NHC$\underline{H}_2$), 2.68-2.81 (m, 2H, H_3, H_6), 3.15 (app d, *J* = 11.8, 1H, H_6), 3.44 (dd, *J* = 11.6, 3.7, 1H, H_2), 3.80 (s, 3H, CH_3), 6.87 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.18 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.2 (CH_{2cpr}), 3.5 (CH_{2cpr}), 11.4 (CH_{cpr}), 34.7 (C_5), 46.8 (C_6), 48.8 (C_4), 52.1 (C_2), 53.0 (NCH_2), 55.4 (CH_3), 60.0 (C_3), 114.3 ($C_{3'}$, $C_{5'}$), 128.8 ($C_{2'}$, $C_{6'}$), 134.9 ($C_{1'}$), 158.6 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 7.18 ppm (d, $H_{2'}$, $H_{6'}$) yielded NOE on 2.68-2.81 (m, H_3). HPLC (t_R, min): 3.39. MS (ESI, *m/z*, %): 261.2 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{16}H_{24}N_2O{\cdot}2HCl{\cdot}H_2O$: %C 54.70, %H 8.03, %N 7.97; experimental: %C 54.84, %H 7.71, %N 7.72.

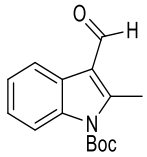
(3*R*,4*R*)-**3** (**3b**). Following general procedure A (work-up B), compound **3b** was obtained from **32b** (70 mg, 0.19 mmol) as an oil (36 mg, 85%, 96% *ee*).



$[\alpha]^{D}_{20}$= +26.7 (c = 1.00, $CHCl_3$). Chiral HPLC (method G, t_R, min): 9.35. Elemental analysis calculated for $C_{16}H_{24}N_2O \cdot 2HCl \cdot H_2O$: %C 54.70, %H 8.03, %N 7.97; experimental: %C 55.31, %H 7.80, %N 7.82. Spectroscopic data were in agreement with those described for enantiomer **3a**.

*4.1.3. Synthesis of tetrahydrocarbazole scaffold. Final compound **4***

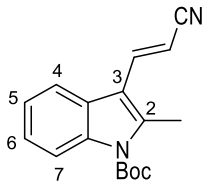
***tert*-Butyl 3-formyl-2-methyl-1*H*-indole-1-carboxylate, 33.**[81] To a solution of 2-methyl-1*H*-indole-3-carbaldehyde (1.71 g, 10.74 mmol) in anhydrous ACN (40 mL), DMAP (131 mg, 1.07 mmol) and di-*tert*-butyl dicarbonate (2.81 g, 12.90 mmol) were added and the reaction mixture was stirred at rt for 4 h. The solvent was removed and the residue was dissolved in $CHCl_3$ and washed with a sat. NaHCO3 solution (x2). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by recrystallization from methanol to afford intermediate **33** as a pale yellow solid (2.54 g, 90%). The spectroscopic data were consistent with those previously reported.[81]



M.p.: 111-112 °C (lit.[81] 110 °C). R_f: 0.43 (hexane/EtOAc 8:2). ^{1}H-NMR ($CDCl_3$): δ 1.72 (s, 9H, $3CH_3$), 2.93 (s, 3H, CH_3), 7.30-7.36 (m, 2H, $2CH_{Ar}$), 8.03-8.09 (m, 1H, CH_{Ar}), 8.28-8.34 (m, 1H, CH_{Ar}), 10.33 (s, 1H, CHO).

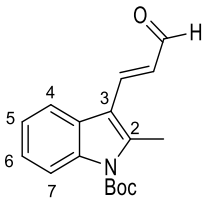
***tert*-Butyl 3-[(*E*)-2-cyanoethenyl]-2-methyl-1*H*-indole-1-carboxylate, 34**.[69] To a solution of diethyl cyanomethylphosphonate (0.24 mL, 1.51 mmol) in anhydrous THF (2.5 mL) at 0 ºC, BuLi (0.59 mL, 2.35 M in hexane) was added and the mixture was stirred at this temperature for 1 h. Next, a solution of **33** (300 mg, 1.16 mmol) in anhydrous THF (1.7 mL) was added dropwise at 0 ºC and the reaction was stirred for 4 h at the same temperature. Then, the reaction was quenched by addition of sat. NH_4Cl solution and concentrated under reduced

pressure. The residue was suspended in water and extracted with EtOAc (x3). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography using hexane to hexane/EtOAc 9:1, to yield title compound **34** as a solid (279 mg, 85%).



M.p.: 119-121 °C. R_f: 0.43 (hexane/EtOAc 8:2). ^{1}H-NMR ($CDCl_3$): δ 1.70 (s, 9H, $3CH_3$), 2.69 (s, 3H, CH_3), 5.92 (d, *J* = 16.6, 1H, CHCN), 7.28-7.36 (m, 2H, H_5, H_6), 7.59 (d, *J* = 16.6, 1H, CHC_{ind}), 7.62-7.69 (m, 1H, H_4), 8.12-8.18 (m, 1H, H_7). ^{13}C-NMR ($CDCl_3$): δ 14.5 (CH_3), 28.4 ($3CH_3$), 85.4 ($\underline{C}(CH_3)_3$), 94.8 ($\underline{C}HCN$), 114.5 (C_3), 115.9 (C_7), 119.1 (C_4), 119.5 (CN), 124.0, 124.8 (C_5, C_6), 126.4 (C_{3a}), 136.4 (C_{8a}), 141.2 (C_2), 142.5 ($\underline{C}HC_{ind}$), 150.1 (CO).

***tert*-Butyl 2-methyl-3-[(1*E*)-3-oxoprop-1-en-1-yl]-1*H*-indole-1-carboxylate, 35.**[69] A solution of **34** (507 mg, 1.80 mmol) in anhydrous toluene (10 mL) was cooled down to -78 ºC, a solution of DIBALH (2.15 mL, 1 M in hexane) was added dropwise for about 10 min and the reaction mixture was stirred at this temperature for 4 h. Then, the reaction was quenched with methanol at -78 ºC and was allowed to warm up to rt. Next, 1 M HCl and a sat. Rochelle salt solution were added and the mixture was stirred for 30 min. The aqueous layer was extracted with EtOAc (x2). The combined organic layers were washed with brine, dried over Na_2SO_4 and filtered, and concentrated under reduced pressure to afford product **35** as an oil (280 mg, 89%). The spectroscopic data were consistent with those previously reported.[69]

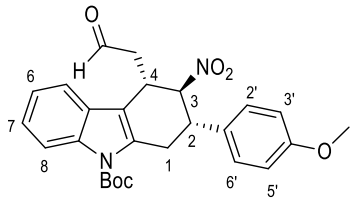


R_f: 0.31 (hexane/EtOAc 8:2). ^{1}H-NMR ($CDCl_3$): δ 1.71 (s, 9H, $3CH_3$), 2.77 (s, 3H, CH_3), 6.87 (dd, *J* = 15.9, 7.7, 1H, $C\underline{H}CHO$), 7.30-7.36 (m, 2H, H_5, H_6), 7.72 (d,

J = 15.9, 1H, CHC_{ind}), 7.80-7.83 (m, 1H, H_4), 8.13-8.16 (m, 1H, H_7), 9.69 (d, *J* = 7.7, 1H, CHO).

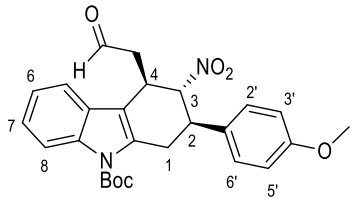
***tert*-Butyl 2-(4-methoxyphenyl)-3-nitro-4-(2-oxoethyl)-1,2,3,4-tetrahydro-9*H*-carbazole-9-carboxylate, 36**.[69] To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq) in toluene (2 mL/mmol), benzoic acid (0.20 eq) was added and the mixture was stirred at rt for 10 min under air. Then, a solution of **35** (1.50 eq) in toluene (2 mL/mmol) and *trans*-4-methoxy-*β*-nitrostyrene (1.00 eq) were added and the reaction was stirred at 70 ºC for 40 h. Afterward, the crude was flushed through a short plug of silica, using a 1:1 mixture of DCM/diethyl ether, and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatopraphy (hexane to hexane/EtOAc 8:2) to afford title compound (2*S*,3*S*,4*S*)- or (2*R*,3*R*,4*R*)-**36**.

(2*S*,3*S*,4*S*)-**36** (**36a**). Following the previous procedure, compound **36a** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (213 mg, 1.18 mmol), using the *S* enantiomer of the catalyst (77 mg, 0.24 mmol), as a yellow solid (294 mg, 53%). The spectroscopic data were consistent with those previously reported.[69]



R_f: 0.30 (hexane/EtOAc 8:2). $[\alpha]^D_{20}$ = -15.9 (c = 0.97, $CHCl_3$) (lit.[69] $[\alpha]^D_{20}$ = -12.01 (c = 0.85, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 1.64 (s, 9H, $3CH_3$), 2.97 (dd, *J* = 18.7, 2.2, ½C<u>H</u>$_2$CHO), 3.25-3.38 (m, 2H, H_1, ½C<u>H</u>$_2$CHO), 3.52-3.62 (m, 2H, H_1, H_2), 3.79 (s, 3H, OCH_3), 4.13-4.21 (m, 1H, H_4), 5.21 (dd, *J* = 10.7, 9.1, H_3), 6.87 (d, *J* = 8.7, $H_{3'}$, $H_{5'}$), 7.22 (d, *J* = 8.8, $H_{2'}$, $H_{6'}$), 7.21-7.33 (m, 2H, H_6, H_7), 7.39 (d, *J* = 7.8, H_5), 8.14 (d, *J* = 7.8, H_8), 9.71 (s, 1H, CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 5.21 ppm (dd, H_3) yielded NOE on 2.97(dd, ½C<u>H</u>$_2$CHO), and 7.22 (d, $H_{2'}$, $H_{6'}$).

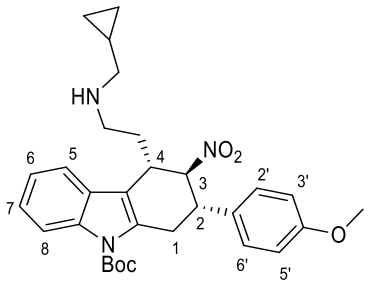
(2*R*,3*R*,4*R*)-**36** (**36b**). Following the previous procedure, compound **36b** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (168 mg, 0.93 mmol), using the *R* enantiomer of the catalyst (61 mg, 0.19 mmol), as a yellow solid (190 mg, 45%).



$[\alpha]^{D}_{20}$= +11.8 (c = 1.31, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **36a**.

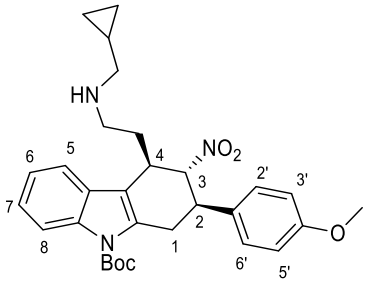
***tert*-Butyl 4-{2-[(cyclopropylmethyl)amino]ethyl}-2-(4-methoxyphenyl)-3-nitro-1,2,3,4-tetrahydro-9*H*-carbazole-9-carboxylate, 37**

(2*S*,3*S*,4*S*)-**37** (**37a**). Following general procedure B, compound **37a** was obtained from **36a** (225 mg, 0.48 mmol) and (cyclopropylmethyl)amine (80 µL, 0.97 mmol) as an oil (218 mg, 86%), which was used in the next step without further purification.



R_f: 0.48 (DCM/ethanol 9:1). $[\alpha]^{D}_{20}$ = -13.8 (c = 1.00, $CHCl_3$). IR (ATR): ν 1730 (C=O), 1550 (NO_2), 1251 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.02-0.07 (m, 2H, CH_{2cpr}), 0.39-0.45 (m, 2H, CH_{2cpr}), 0.80-0.93 (CH_{cpr}), 1.64 (s, 9H, $3CH_3$), 1.96-2.07 (m, 1H, ½$NHCH_2C\underline{H}_2$), 2.29-2.43 (m, 3H, ½$NHCH_2C\underline{H}_2$, $NHC\underline{H}_2CH$), 2.46-2.55 (m, 1H, ½$NHC\underline{H}_2CH_2$), 2.61-2.70 (m, 1H, ½$NHC\underline{H}_2CH_2$), 3.18-3.28 (m, 1H, H_1), 3.42-3.56 (m, 2H, H_1, H_2), 3.79 (s, 3H, OCH_3), 3.88-3.93 (m, 1H, H_4), 5.25-5.32 (m, 1H, H_3), 6.87 (d, *J* = 8.7, $H_{3'}$, $H_{5'}$), 7.20-7.33 (m, 2H, H_6, H_7), 7.21 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 7.59 (d, *J* = 7.1, 1H, H_5), 8.12 (d, *J* = 7.7, 1H, H_8). ^{13}C-NMR ($CDCl_3$): δ 3.4 (CH_{2cpr}), 11.2 (CH_{cpr}), 28.4 ($3CH_3$), 30.6 ($NHCH_2\underline{C}H_2$), 32.9 (C_1), 38.5 (C_4), 45.4 ($NHC\underline{H}_2CH_2$), 45.5 (C_2), 54.9 ($NH\underline{C}H_2CH$), 55.4 (OCH_3), 84.4 ($\underline{C}(CH_3)_3$), 93.6 (C_3), 114.4 ($C_{3'}$, $C_{5'}$), 115.3 (C_{4a}), 115.8 (C_8), 119.1 (C_5), 123.1, 124.2 (C_6, C_7), 127.9 (C_{8a}), 128.8 ($C_{2'}$, $C_{6'}$), 131.1 ($C_{1'}$), 134.3 (C_{9a}), 136.3 (C_{4b}), 150.4 (NCOO), 159.3 ($C_{4'}$). HPLC (t_R, min): 19.58. MS (ESI, *m/z*, %): 520.0 ($[M+H]^+$, 100).

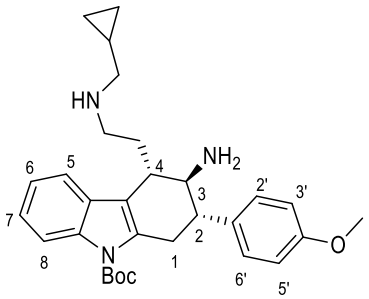
(2*R*,3*R*,4*R*)-**37** (**37b**). Following general procedure B, compound **37b** was obtained from **36b** (200 mg, 0.43 mmol) and (cyclopropylmethyl)amine (75 µL, 0.86 mmol) as an oil (186 mg, 83%), which was used in the next step without further purification.



$[\alpha]^{D}_{20}$= +12.13 (c = 0.75, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **37a**.

***tert*-Butyl 3-amino-4-{2-[(cyclopropylmethyl)amino]ethyl}-2-(4-methoxyphenyl)-1,2,3,4-tetrahydro-9*H*-carbazole-9-carboxylate, 38**

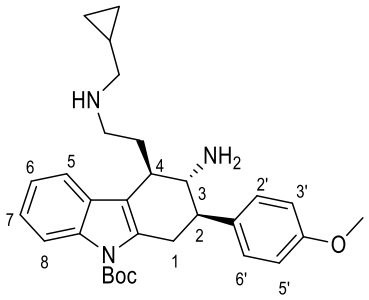
(2*S*,3*S*,4*S*)-**38** (**38a**). Following general procedure C, compound **38a** was obtained from **37a** (218 mg, 0.42 mmol) as an oil (220 mg, quantitative), which was used in the next step without further purification.



R_f: 0.34 (DCM/methanol/NH_3 9:1:0.1). $[\alpha]^{D}_{20}$ = -15.1 (c = 1.00, $CHCl_3$). IR (ATR): ν 1727 (C=O), 1363 (C-N), 1249 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.03-0.08 (m, 2H, CH_{2cpr}), 0.40-0.46 (m, 2H, CH_{2cpr}), 0.84-0.98 (m, 1H, CH_{cpr}), 1.62 (s, 9H, $3CH_3$), 2.17-2.27 (m, 1H, ½NHCH$_2$C<u>H</u>$_2$), 2.31-2.36 (m, 1H, ½NHCH$_2$C<u>H</u>$_2$), 2.41 (d, *J* = 6.9, 2H, NHC<u>H</u>$_2$CH), 2.53-2.62 (m, 1H, ½NHC<u>H</u>$_2$CH$_2$), 2.64-2.82 (m, 2H, H_2, ½NHC<u>H</u>$_2$CH$_2$), 2.92-2.97 (m, 1H, H_4), 3.09 (ddd, *J* = 18.0, 11.3, 2.6, 1H, H_1), 3.19 (dd, *J* = 10.5, 8.5, 1H, H_3), 3.35 (dd, *J* = 17.9, 4.3, 1H, H_1), 3.82 (s, 3H, OCH_3) 6.91 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.19-7.28 (m, 2H, H_6, H_7), 7.22 (d, *J* = 8.4, 2H, $H_{2'}$, $H_{6'}$), 7.57 (dd, *J* = 6.8, 2.1, 1H, H_5), 8.11 (dd, *J* = 7.1, 1.9, 1H, H_8). ^{13}C-NMR ($CDCl_3$): δ

3.5 ($2CH_{2cpr}$), 11.1 (CH_{cpr}), 28.4 ($3CH_3$), 32.1 ($NHCH_2\underline{C}H_2$), 33.7 (C_1), 41.4 (C_4), 46.9 ($NH\underline{C}H_2CH_2$), 49.7 (C_2), 55.1 ($NH\underline{C}H_2CH$), 55.4 (OCH_3), 56.5 (C_3), 83.8 ($\underline{C}(CH_3)_3$), 114.4 ($C_{3'}$, $C_{5'}$), 115.6 (C_8), 118.4 (C_{4a}), 119.0 (C_5), 122.7 (C_6), 123.5 (C_7), 128.9 (C_{4b}), 129.1 ($C_{2'}$, $C_{6'}$), 135.2 (C_{9a}), 135.4 ($C_{1'}$), 136.4 (C_{8a}), 150.6 (NCOO), 158.7 ($C_{4'}$). HPLC (t_R, min): 13.78. MS (ESI, *m/z*, %): 490.1 ($[M+H]^+$, 100).

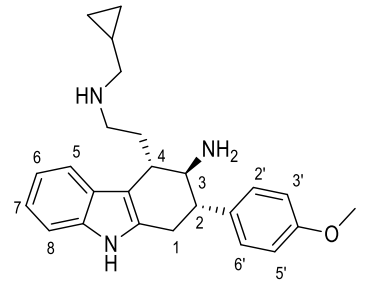
(2*R*,3*R*,4*R*)-**38** (**38b**). Following general procedure C, compound **38b** was obtained from **37b** (160 mg, 0.31 mmol) as an oil (152 mg, quantitative), which was used in the next step without further purification.



$[\alpha]^{D}_{20}$= +17.8 (c = 1.03, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **38a**.

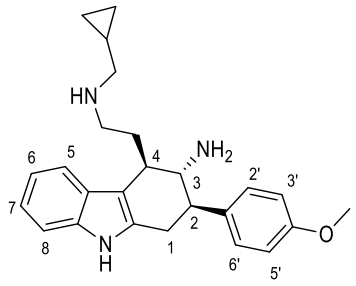
4-{2-[(Cyclopropylmethyl)amino]ethyl}-2-(4-methoxyphenyl)-2,3,4,9-tetrahydro-1*H*-carbazol-3-amine, 4. To a solution of enantiomer **38a** or **38b** (1.00 eq) in methanol (2.5 mL/mmol), HCl (35.0 eq, 37% aq. solution) was added and the reaction mixture was stirred at rt for 4 h. Then, solvents were evaporated under reduced pressure and the resulting solid was washed with anhydrous diethyl ether and dried under vacuum to afford the corresponding hydrochloride salt of (2*S*,3*S*,4*S*) or (2*R*,3*R*,4*R*)-**4**.

(2*S*,3*S*,4*S*)-**4** (**4a**). Following the previous procedure, compound **4a** was obtained from **38a** (85 mg, 0.17 mmol) as hydrochloride salt (42 mg, 50%, 93% *ee*).



R_f: 0.30 (DCM/methanol/NH_3 9:1:0.1). $[\alpha]^D_{20}$ = -5.4 (c = 0.24, methanol). Chiral HPLC (method J, t_R, min): 9.74. IR (ATR): ν 3375 (NH), 1458 (C-N). ^{1}H-NMR (methanol-*d4*): δ 0.26-0.36 (m, 2H, CH_{2cpr}), 0.55-0.65 (m, 2H, $2CH_{2cpr}$), 0.93-1.02 (m, 1H, CH_{cpr}), 2.21-2.43 (m, 1H, $NHCH_2C\underline{H}_2$), 2.64-2.82 (m, 3H, $NHC\underline{H}_2CH$, ½$NHC\underline{H}_2CH_2$), 3.01-3.24 (m, 3H, $2H_1$, ½$NHC\underline{H}_2CH_2$), 3.29-3.38 (m, 1H, H_2), 3.48-3.55 (m, H_4), 3.79 (s, 3H, CH_3), 3.92-3.97 (m, 1H, H_3), 6.96 (d, *J* = 8.3, 2H, $H_{3'}$, $H_{5'}$), 7.01-7.11 (m, 2H, H_6, H_7), 7.32 (d, *J* = 7.7, 1H, H_8), 7.37 (d, *J* = 8.5, 2H, $H_{2'}$, $H_{6'}$), 7.56 (d, *J* = 7.7, 1H, H_5). ^{13}C-NMR (methanol-*d4*): δ 4.4 (CH_{2cpr}), 4.6 (CH_{2cpr}), 8.13 (CH_{cpr}), 27.8 ($NHCH_2\underline{C}H_2$), 29.4 (C_1), 37.6 (C_4), 44.4 (C_2), 45.1 ($NH\underline{C}H_2CH_2$), 53.7 ($NH\underline{C}H_2CH$), 55.8 (CH_3), 56.1 (C_3), 106.7 (C_{4a}), 112.3 (C_5), 115.8 ($C_{3'}$, $C_{5'}$), 118.8 (C_8), 120.4 (C_6), 122.5 (C_7), 127.4 (C_{4b}), 130.3 ($C_{2'}$, $C_{6'}$), 132.7 ($C_{1'}$), 134.8 (C_{9a}), 138.3 (C_{8a}), 161.0 ($C_{4'}$). HPLC (t_R, min): 11.45. MS (ESI, *m/z*, %): 390.1 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{25}H_{31}N_3O{\cdot}2HCl{\cdot}2H_2O$: %C 60.24, %H 7.98, %N 8.43; experimental: %C 60.98, %H 7.74, %N 8.11.

(2*R*,3*R*,4*R*)-**4** (**4b**). Following the previous procedure, compound **4b** was obtained from **38b** (100 mg, 0,2 mmol) as hydrochloride salt (40 mg, 40%, 93% *ee*).



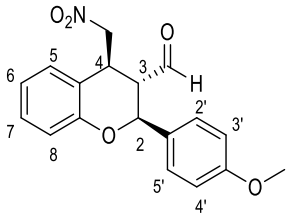
$[\alpha]^D_{20}$= +6.8 (c = 0.30, methanol). Chiral HPLC (method J, t_R, min): 19.77. Elemental analysis calculated for $C_{25}H_{31}N_3O{\cdot}2HCl{\cdot}2H_2O$: %C 60.24, %H 7.98, %N 8.43; experimental: %C 61.24, %H 7.68, %N 8.15. Spectroscopic data were in agreement with those described for enantiomer **4a**.

*4.1.4. Synthesis of chromane and tetrahydrobenzo[c]chromene scaffolds. Final compounds **5**-**9** and **46**-**48***

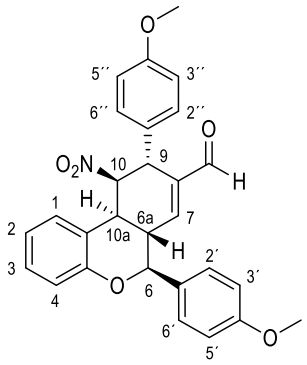
2-(4-Methoxyphenyl)-4-(nitromethyl)-3,4-dihydro-2*H*-1-benzopyran-3-carbaldehyde, 39 and 6,9-bis(4-methoxyphenyl)-10-nitro-6a,9,10,10a-tetrahydro-6*H*-benzo[*c*]chromene-8-carbaldehyde, 40. To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq), *p*-

methoxycinnamaldehyde (1.20 eq) and acetic acid (0.2 eq) in anhydrous chloroform (16.6 mL/mmol), *trans*-2-hydroxy-*β*-nitrostyrene (1 eq) was added and the resulting solution was stirred at 25 ºC overnight. Then, the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (hexane to hexane/EtOAc 8:2) to afford (2*S*,3*S*,4*S*)- or (2*R*,3*R*,4*R*)-**39** and (6*S*,6a*S*,9*R*,10*R*,10a*S*)- or (6*R*,6a*R*,9*S*,10*S*,10a*R*)-**40**, respectively.

(2*S*,3*S*,4*S*)-**39** (**39a**) and (6*S*,6a*S*,9*R*,10*R*,10a*S*)-**40** (**40a**). Following the previous procedure, compounds **39a** and **40a** were obtained from *trans*-2-hydroxy-*β*-nitrostyrene (50 mg, 0.30 mmol), using the *S* enantiomer of the catalyst (19.7 mg, 0.06 mmol), as yellow solids in 31% (31 mg) and 21% (30 mg) yields, respectively.

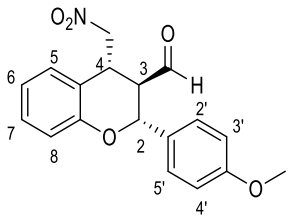


39a: M.p.: 110-112 ºC. R_f: 0.67 (hexane/EtOAc 7:3). $[\alpha]^{D}_{20}$ = -32.20 (c = 1.06, $CHCl_3$). IR (ATR): ν 1724 (C=O), 1585 (NO_2), 1247 (COC). ^{1}H-NMR ($CDCl_3$): δ 3.58 (td, *J* = 8.1, 1.0, 1H, H_3), 3.83 (s, 3H, CH_3), 4.20-4.30 (m, 1H, H_4), 4.50 (dd, *J* = 13.1, 6.9, 1H, ½CH_2NO_2), 4.59 (dd, *J* = 13.1, 4.6, 1H, ½CH_2NO_2), 5.07 (d, *J* = 8.1, 1H, H_2), 6.94-7.04 (m, 2H, H_6, H_8), 6.96 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.22 (app t, *J* = 7.5, 2H, H_5, H_7), 7.35 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 9.49 (d, *J* = 1.0, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 33.7 (C_4), 54.2 (C_3), 55.5 (CH_3), 76.7 (C_2), 77.8 (CH_2NO_2), 114.8 ($C_{3'}$, $C_{5'}$), 118.1 (C_8), 119.1 (C_{4a}), 122.3 (C_6), 127.9 (C_5), 128.1 ($C_{2'}$, $C_{6'}$), 129.26 (C_7), 129.30 ($C_{1'}$), 154.9 (C_{8a}), 160.3 ($C_{4'}$), 199.9 (CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.20-4.30 ppm (m, H_4) yielded NOE on 5.07 (d, H_2), 7.22 (d, H_5), and 9.49 (d, CHO). HPLC (t_R, min): 19.86. MS (ESI, *m/z*, %): 328.1 ($[M+H]^+$, 100).

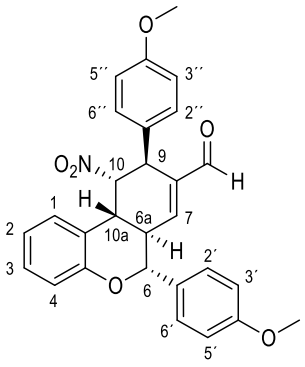


40a: M.p.: 175-177 ºC (lit.[70] 176-179 ºC). R_f: 0.42 (hexane/EtOAc 7:3). $[\alpha]^{D}_{20}$ = +12.0 (c = 0.29, $CHCl_3$) (lit.[70] $[\alpha]^{D}_{25}$: +17.5 (c = 0.30, $CHCl_3$)). ^{1}H-NMR ($CDCl_3$): δ 3.33-3.44 (m, 1H, H_{6a}), 3.58 (dd, *J* = 11.4, 2.2, 1H, H_{10a}), 3.80 (s, 3H, CH_3), 3.89 (s, 3H, CH_3), 4.60 (s, 1H, H_9), 5.07 (d, *J* = 10.1, 1H, H_6), 5.45 (d, *J* = 2.2, 1H, H_{10}), 6.61 (d, *J* = 1.4, 1H, H_7), 6.87-6.92 (m, 4H, $H_{3''}$, $H_{5''}$, H_2, H_4), 7.06 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.13-7.16 (m, 4H, $H_{2''}$, $H_{6''}$, H_1, H_3), 7.48 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 9.37 (s, 1H, CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 5.07 ppm (d, H_6) yielded NOE on 3.58 (dd, H_{10a}); irradiation of the signal at δ 3.58 ppm (dd, H_{10a}) yielded NOE on 5.07 (d, H_6), 5.45 (d, H_{10}), and 7.13-7.16 (m, $H_{2''}$, $H_{6''}$); irradiation of the signal at δ 5.45 ppm (dd, H_{10}) yielded NOE on 3.58 (dd, H_{10a}), and 7.13-7.16 (m, $H_{2''}$, $H_{6''}$); and irradiation of the signal at δ 3.33-3.44 ppm (m, H_{6a}) yielded NOE on 7.48 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 23.48. MS (ESI, *m/z*, %): 472.1 ($[M+H]^+$, 100). The spectroscopic data were consistent with those previously reported.[70]

(2*R*,3*R*,4*R*)-**39** (**39b**) and (6*R*,6a*R*,9*S*,10*S*,10a*R*)-**40** (**40b**). Following the previous procedure, compounds **39b** and **40b** were obtained from *trans*-2-hydroxy-*β*-nitrostyrene (50 mg, 0.30 mmol), using the *R* enantiomer of the catalyst (19.7 mg, 0.06 mmol), as yellow solids in 22% (22 mg) and 26% (37 mg) yields, respectively.

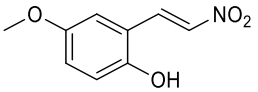


39b: $[\alpha]^{D}_{20}$= +34.60 (c = 1.13, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **39a**.



40b: $[\alpha]^{D}_{20}$= -10.1 (c = 0.28, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **40a**.

4-Methoxy-2-[(*E*)-2-nitroethenyl]phenol, 41.[82] To a solution of 2-hydroxy-5-methoxybenzaldehyde (1.00 mL, 8.0 mmol) and nitromethane (2.20 mL, 40.0 mmol) in acetic acid (6 mL), ammonium acetate (124 mg, 1.6 mmol) was added and the reaction mixture was refluxed overnight. Then, the solvent was evaporated under reduced pressure, and the residue was dissolved in EtOAc, washed with water (x4), a sat. $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography (hexane to hexane/EtOAc 8:2) to afford compound **41** as an orange solid (937 mg, 60%). The spectroscopic data were consistent with those previously reported.[83]

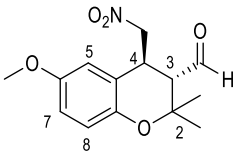


M.p.: 151-152 (lit.[83] 152-153 °C). R_f: 0.28 (hexane/EtOAc 7:3). ^{1}H-NMR ($CDCl_3$): δ 3.80 (s, 3H, CH_3), 5.34 (s, 1H, OH), 6.76-6.80 (m, 1H, CH_{Ar}), 6.90-6.95 (m, 2H, $2CH_{Ar}$), 7.90 (d, *J* = 13.6, 1H, CHC_{Ar}), 8.11 (d, *J* = 13.6, 1H, $CHNO_2$).

6-Methoxy-2,2-dimethyl-4-(nitromethyl)-3,4-dihydro-2*H*-1-benzopyran-3-carbaldehyde, 42. To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq), 3,3-dimethylacrolein (1.50 eq), and acetic acid (0.20 eq) in anhydrous chloroform (5 mL/mmol), **41** (1.00 eq) was added and the reaction was stirred at rt for 1 h. Then, the mixture was diluted with EtOAc and washed with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography (hexane to hexane/EtOAc 9:1) to afford the

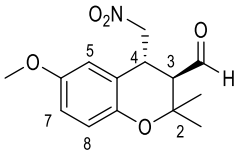
corresponding chromene (3*S*,4*S*)- or (3*R*,4*R*)-**42**.

(3*S*,4*S*)-**42** (**42a**). Following the previous procedure, compound **42a** (dr = 8:2) was obtained from **41** (196 mg, 1.00 mmol), using the *S* enantiomer of the catalyst (65 mg, 0.20 mmol), as a yellow oil (170 mg, 61%).



R_f: 0.49 (hexane/EtOAc 7:3). IR (ATR): ν 1720 (C=O), 1551 (NO_2), 1238 (COC). ^{1}H-NMR ($CDCl_3$, major diastereoisomer): δ 1.12 (CH_3), 1.67 (CH_3), 3.21 (dd, *J* = 10.4, 1.3, 1H, H_3), 3.75 (OCH_3), 3.98 (app dt, *J* = 10.3, 5.1, 1H, H_4), 4.65 (dd, *J* = 13.3, 4.6, 1H, ½CH_2NO_2), 4.72 (dd, *J* = 13.3, 6.0, 1H, ½CH_2NO_2), 6.72-6.73 (m, 1H, H_5), 6.76-6.81 (m, 2H, H_7, H_8), 9.89 (d, *J* = 1.6, 1H, CHO). ^{13}C-NMR ($CDCl_3$, major diastereoisomer): δ 21.3 (CH_3), 28.5 (CH_3), 31.7 (C_4), 55.8 (OCH_3), 57.7 (C_3), 74.4 (C_2), 78.1 (CH_2NO_2), 111.9 (C_5), 115.1, 119.2 (C_7, C_8), 119.7 (C_{4a}), 146.7 (C_{8a}), 154.2 (C_6), 200.3 (CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 3.21 ppm (dd, H_3) yielded NOE on 1.67 (s, CH_3), 4.65 (dd, ½CH_2NO_2), and 4.72 (dd, ½CH_2NO_2); and irradiation of the signal at δ 3.98 ppm (app dt, H_4) yielded NOE on 1.12 (s, CH_3). HPLC (t_R, min): 18.80. MS (ESI, *m/z*, %): 297.2 ($[M+NH_4]^+$, 100).

(3*R*,4*R*)-**42** (**42b**). Following the previous procedure, compound **42b** (dr = 8:2) was obtained from **41** (325 mg, 1.67 mmol), using the *R* enantiomer of the catalyst (108 mg, 0.33 mmol), as a yellow oil (242 mg, 52%).

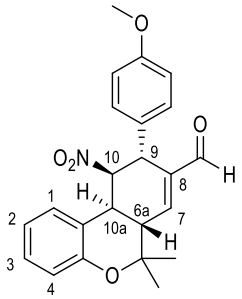


Spectroscopic data were in agreement with those described for enantiomer **42a**.

9-(4-Methoxyphenyl)-6,6-dimethyl-10-nitro-6a,9,10,10a-tetrahydro-6*H*-dibenzo[*b*,*d*]pyran-8-carbaldehyde, 43.[70] To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq), 3,3-dimethylacrolein (1.10 eq), and acetic acid (0.20 eq) in anhydrous chloroform (5 mL/mmol), *trans*-

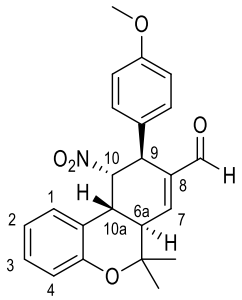
2-hydroxy-β-nitrostyrene (1.00 eq) was added and the reaction was stirred at rt for 1 h. Next, *p*-methoxycinnamaldehyde (1.20 eq) was added and the reaction mixture was stirred at rt for 24 h. Then, the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (hexane to hexane/EtOAc 85:15) to afford corresponding enantiomer (6a*S*,9*R*,10*R*,10a*S*)- or (6a*R*,9*S*,10*S*,10a*R*)-**43**.

(6a*S*,9*R*,10*R*,10a*S*)-**43** (**43a**). Following the previous procedure, compound **43a** was obtained from *trans*-2-hydroxy-β-nitrostyrene (100 mg, 0.40 mmol), using the *S* enantiomer of the catalyst (22 mg, 0.08 mmol), as a yellow oil (80 mg, 48%). The spectroscopic data were consistent with those previously reported.[70]



R_f: 0.35 (hexane/EtOAc 7:3). $[\alpha]^D_{20}$ = -86.8 (c = 2.00, $CHCl_3$) (lit.[70] $[\alpha]^D_{20}$ = -83.1 (c = 2.00, $CHCl_3$). IR (ATR): ν 1691 (C=O), 1546 (NO_2), 1489 (C-N), 1253 (COC). ^{1}H-NMR ($CDCl_3$): δ 1.31 (s, 3H, CH_3), 1.76 (s, 3H, CH_3), 3.06 (d, *J* = 12.2, 1H, H_{6a}), 3.35 (dd, *J* = 12.2, 1H, H_{10a}), 3.79 (s, 3H, OCH_3), 4.60 (s, 1H, H_9), 5.47 (s, 1H, H_{10}), 6.81-6.90 (m, 4H, H_7, $3CH_{Ar}$), 7.11-7.17 (m, 5H, $5CH_{Ar}$), 9.60 (s, 1H, CHO).

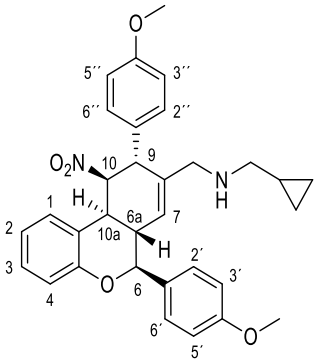
(6a*R*,9*S*,10*S*,10a*R*)-**43** (**43b**). Following the previous procedure, compound **43b** was obtained from *trans*-2-hydroxy-β-nitrostyrene (180 mg, 0.72 mmol), using the *R* enantiomer of the catalyst (47 mg, 0.14 mmol), as a yellow oil (122 mg, 43%).



$[\alpha]^D_{20}$= +86.1 (c = 1.98, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **43a**.

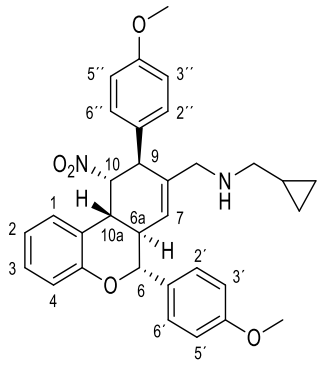
1-[6,9-Bis(4-methoxyphenyl)-10-nitro-6a,9,10,10a-tetrahydro-6*H*-benzo-[*c*]chromen-8-yl]-*N*-(cyclopropylmethyl)methanamine, 44.

(6*S*,6a*S*,9*R*,10*R*,10a*S*)-**44** (**44a**). Following general procedure B, compound **44a** was obtained from **40a** (73 mg, 0.16 mmol) and (cyclopropylmethyl)amine (28 µL, 0.32 mmol) as a yellow oil (83 mg, quantitative), which was used in the next step without further purification.



R_f: 0.61 (DCM/methanol 9:1). $[\alpha]^D_{20}$ = +25.7 (c = 0.61, $CHCl_3$). IR (ATR): ν 1610 (C=C), 1548 (NO_2), 1250 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.07-0.17 (m, 3H, ½CH_{2cpr}, CH_{2cpr}), 0.26-0.35 (m, 1H, ½CH_{2cpr}), 0.80-0.93 (m, 1H, CH_{cpr}), 2.29 (dd, *J* = 12.7, 7.9, 1H, ½NHC$\underline{H}_2$CH), 2.72 (dd, *J* = 12.4, 6.8, 1H, ½NHC$\underline{H}_2$CH), 2.96 (t, *J* = 11.0, 1H, H_{6a}), 3.13 (d, *J* = 13.7, 1H, ½NHC$\underline{H}_2$), 3.35 (d, *J* = 13.7, 1H, ½NHC$\underline{H}_2$), 3.50 (d, *J* = 11.6, 1H, H_{10a}), 3.79 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 4.81 (s, 1H, H_9), 4.93 (d, *J* = 10.4, 1H, H_6), 5.36 (s, 1H, H_{10}), 5.60 (s, 1H, H_7), 6.83-6.91 (m, 2H, H_2, H_4), 6.89 (d, *J* = 8.7, 2H, $H_{3''}$, $H_{5''}$), 7.00 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.11-7.19 (m, 2H, H_1, H_3), 7.28 (d, *J* = 8.7, 2H, $H_{2''}$, $H_{6''}$), 7.40 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 4.1 (CH_{2cpr}), 4.4 (CH_{2cpr}), 8.1 (CH_{cpr}), 35.6 (C_{10a}), 38.6 (C_{6a}), 44.8 (C_9), 50.9 ($NHCH_2$), 51.1 (NH$\underline{C}H_2$CH), 55.51 (CH_3), 55.53 (CH_3), 82.5 (C_6), 85.8 (C_{10}), 114.5 ($C_{3'}$, $C_{5'}$), 114.8 ($C_{3''}$, $C_{5''}$), 117.2 (C_4), 119.2 (C_{10b}), 120.6 (C_2), 125.2 (C_1), 128.9 (C_3), 129.1 ($C_{2'}$, $C_{6'}$), 129.8 ($C_{1''}$), 130.2 ($C_{1'}$), 130.4 (C_7, $C_{2''}$, $C_{6''}$), 131.0 (C_8), 155.1 (C_{4a}), 159.6 ($C_{4''}$), 160.4 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 2.96 ppm (t, H_{6a}) yielded NOE on 4.81 (s, H_9), and 7.40 (d, $H_{2'}$, $H_{6'}$); irradiation of the signal at δ 3.50 ppm (d, H_{10a}) yielded NOE on 4.93 (d, H_6), 5.36 (s, H_{10}), and 7.28 (d, $H_{2''}$, $H_{6''}$); irradiation of the signal at δ 4.93 ppm (d, H_6) yielded NOE on 3.50 (d, H_{10a}); and irradiation at δ 5.36 ppm (s, H_{10}) yielded NOE on 3.50 (d, H_{10a}), and 7.28 (d, $H_{2''}$, $H_{6''}$). HPLC (t_R, min): 12.3. MS (ESI, *m/z*, %): 419.1 ($[M+H]^+$, 100).

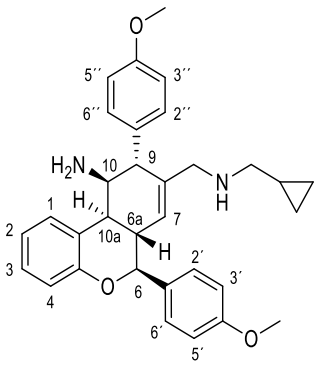
(6*R*,6a*R*,9*S*,10*S*,10a*R*)-**44** (**44b**). Following general procedure B, compound **44b** was obtained from **40b** (87 mg, 0.18 mmol) and (cyclopropylmethyl)amine (31 µL, 0.36 mmol) as a yellow oil (96 mg, quantitative), which was used in the next step without further purification.



$[\alpha]^{D}_{20}$= -22.5 (c = 0.47, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **44a**.

8-{[(Cyclopropylmethyl)amino]methyl}-6,9-bis(4-methoxyphenyl)-6a,9,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-10-amine, 8

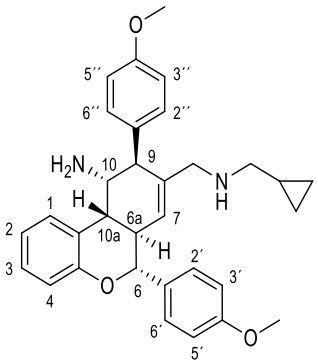
(6*S*,6a*S*,9*R*,10*R*,10a*S*)-**8** (**8a**). Following general procedure C, compound **8a** was obtained from **44a** (83 mg, 0.16 mmol) as an oil (33 mg, 42%, 99% *ee*). Chromatography: DCM to DCM/methanol 9:1.



R_f: 0.32 (DCM/methanol 9:1). $[\alpha]^{D}_{20}$ = +25.4 (c = 0.90, $CHCl_3$). Chiral HPLC (method C, t_R, min): 29.3. IR (ATR): ν 1610 (C=C), 1245 (COC). ^{1}H-NMR ($CDCl_3$): δ -0.05-(-0.02) (m, 2H, CH_{2cpr}), 0.34-0.42 (m, 2H, CH_{2cpr}), 0.77-0.90 (m, 1H, CH_{cpr}), 2.26-2.36 (m, 2H, NHC$\underline{H}_2$CH), 2.80 (t, *J* = 10.8, 1H, H_{6a}), 3.04 (s, 2H, NHC$\underline{H}_2$), 3.08 (d, *J* = 11.8, 1H, H_{10a}), 3.62 (s, 1H, H_9), 3.74 (s, 1H, H_{10}), 3.80 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 4.99 (d, *J* = 10.8, 1H, H_6), 5.43 (s, 1H, H_7), 6.81-6.91 (m, 2H, H_2, H_4), 6.88 (d, *J* = 8.7, 2H, $H_{3''}$, $H_{5''}$), 6.99 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.04 (d, *J* = 7.9, 1H, H_1), 7.10 (t, *J* = 7.8, 1H, H_3), 7.20 (d, *J* = 8.7, 2H, $H_{2''}$, $H_{6''}$), 7.43 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.4 (CH_{2cpr}), 3.6 (CH_{2cpr}), 10.9 (CH_{cpr}), 36.2 (C_{10a}), 37.3 (C_{6a}), 50.4 (C_9), 52.9 (C_{10}), 53.8 ($NHCH_2$), 54.4 (NH$\underline{C}H_2$CH), 55.4 (CH_3), 55.5

(CH_3), 83.0 (C_6), 114.17 ($C_{3''}$, $C_{5''}$), 114.25 ($C_{3'}$, $C_{5'}$), 116.9 (C_4), 120.4 (C_2), 122.4 (C_7), 123.1 (C_{10b}), 124.5 (C_1), 128.0 (C_3), 129.2 ($C_{2'}$, $C_{6'}$), 129.7 ($C_{2''}$, $C_{6''}$), 131.7 ($C_{1'}$), 134.2 ($C_{1''}$), 156.1 (C_{4a}), 158.6 ($C_{4''}$), 160.0 ($C_{4'}$), C_8 not observed. 1D ^{1}H-NMR NOE: irradiation of the signal at δ 3.74 ppm (s, H_{10}) yielded NOE on 3.08 (d, H_{10a}), and 7.20 (d, $H_{2''}$, $H_{6''}$); irradiation of the signal at δ 3.50 ppm (d, H_{10a}) yielded NOE on 4.93 (d, H_6), 5.36 (s, H_{10}), and 7.28 (d, $H_{2''}$, $H_{6''}$); irradiation of the signal at δ 4.93 ppm (d, H_6) yielded NOE on 3.50 (d, H_{10a}); and irradiation of the signal at δ 5.36 ppm (s, H_{10}) yielded NOE on 3.50 (d, H_{10a}), and 7.28 (d, $H_{2''}$, $H_{6''}$). HPLC (t_R, min): 13.71. MS (ESI, *m/z*, %): 497.3 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{32}H_{36}N_2O_3 \cdot 2HCl \cdot H_2O$: %C 65.41, %H 6.86, %N 4.77; experimental: %C 65.47, %H 6.55, %N 4.59.

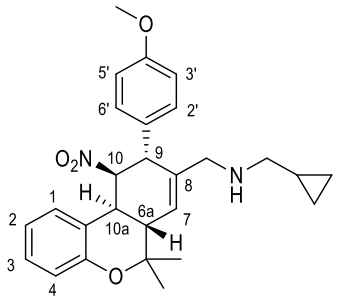
(6*R*,6a*R*,9*S*,10*S*,10a*R*)-**8** (**8b**). Following general procedure C, compound **8b** was obtained from **44b** (81 mg, 0.15 mmol) as an oil (31 mg, 41%, 99% *ee*). Chromatography: DCM to DCM/methanol 9:1.



$[\alpha]^D_{20}$= -27.1 (c = 0.86, $CHCl_3$). Chiral HPLC (method C, t_R, min): 23.1. Elemental analysis calculated for $C_{32}H_{36}N_2O_3 \cdot 2HCl \cdot H_2O$: %C 65.41, %H 6.86, %N 4.77; experimental: %C 65.74, %H 6.54, %N 4.56. Spectroscopic data were in agreement with those described for enantiomer **8a**.

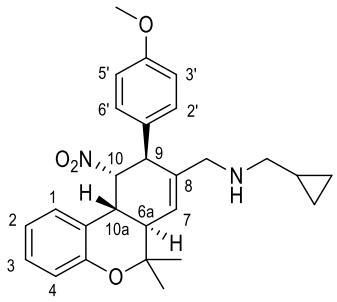
1-Cyclopropyl-*N*-{[9-(4-methoxyphenyl)-6,6-dimethyl-10-nitro-6a,9,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-8-yl]methyl}methanamine, 45

(6a*S*,9*R*,10*R*,10a*S*)-**45** (**45a**). Following general procedure B, compound **45a** was obtained from **43a** (105 mg, 0.27 mmol) and (cyclopropylmethyl)amine (47 µL, 0.54 mmol) as a yellow oil (108 mg, 90%), which was used in the next step without further purification.



R_f: 0.64 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^D_{20}$ = -38.8 (c = 1.06, $CHCl_3$). IR (ATR): ν 1609 (C=C), 1546 (NO_2), 1252 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.06-0.10 (m, 2H, CH_{2cpr}), 0.41-0.47 (m, 2H, CH_{2cpr}), 0.84-0.93 (m, 1H, CH_{cpr}), 1.25 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 2.35 (dd, *J* = 12.1, 6.8, 1H, ½NHC$\underline{H}_2$CH), 2.44 (dd, *J* = 12.1, 6.9, 1H, ½NHC$\underline{H}_2$CH), 2.78 (d, *J* = 12.2, 1H, H_{6a}), 3.16 (s, 2H, NHC$\underline{H}_2$), 3.24 (dd, *J* = 12.2, 2.7, 1H, H_{10a}), 3.80 (s, 3H, OCH_3), 4.32 (s, 1H, H_9), 5.38 (d, *J* = 2.7, 1H, H_{10}), 6.02 (s, 1H, H_7), 6.77-6.83 (m, 2H, H_2, H_4), 6.91 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.07-7.13 (m, 2H, H_1, H_3), 7.23 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.5 (CH_{2cpr}), 3.6 (CH_{2cpr}), 11.3 (CH_{cpr}), 21.7 (CH_3), 28.2 (CH_3), 31.8 (C_{10a}), 41.0 (C_{6a}), 46.0 (C_9), 53.6 ($NHCH_2$), 54.2 (NH$\underline{C}H_2$CH), 55.5 (OCH_3), 77.9 (C_6), 86.9 (C_{10}), 114.8 ($C_{3'}$, $C_{5'}$), 117.6 (C_4), 119.1 (C_{10b}), 119.8 (C_2), 123.8 (C_7), 124.8 (C_1), 128.6 (C_3), 129.9 ($C_{2'}$, $C_{6'}$), 131.5 ($C_{1'}$), 136.8 (C_8), 153.9 (C_{4a}), 159.4 ($C_{4'}$). HPLC (t_R, min): 16.7. MS (ESI, *m/z*, %): 499.0 ($[M+H]^+$, 100).

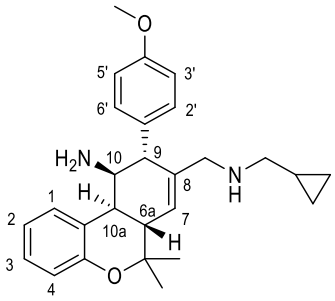
(6a*R*,9*S*,10*S*,10a*R*)-**45** (**45b**). Following general procedure B, compound **45b** was obtained from **43b** (120 mg, 0.31 mmol) and (cyclopropylmethyl)amine (54 µL, 0.62 mmol) as a yellow oil (120 mg, 88%), which was used in the next step without further purification.



$[\alpha]^D_{20}$= +36.1 (c = 1.02, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **45a**.

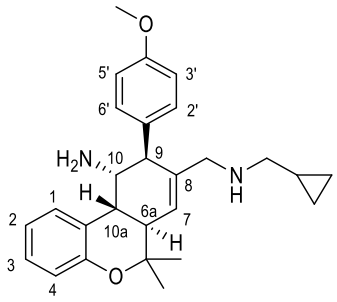
8-{[(Cyclopropylmethyl)amino]methyl}-9-(4-methoxyphenyl)-6,6-dimethyl-6a,9,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-10-amine, 9

(6a*S*,9*R*,10*R*,10a*S*)-**9** (**9a**). Following general procedure C, compound **9a** was obtained from **45a** (108 mg, 0.24 mmol) as a yellow oil (60 mg, 60%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.05.



R_f: 0.32 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^{D}_{20}$ = -72.4 (c = 0.78, $CHCl_3$). Chiral HPLC (method D, t_R, min): 9.2. IR (ATR): ν 2925 (NH), 1609 (C=C), 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.05-0.10 (m, 2H, CH_{2cpr}), 0.42-0.48 (m, 2H, CH_{2cpr}), 0.83-0.95 (m, 1H, CH_{cpr}), 1.27 (s, 3H, CH_3), 1.63 (s, 3H, CH_3), 2.36 (dd, *J* = 12.0, 6.8, 1H, ½ NHC$\underline{H}_2$CH), 2.42-2.49 (m, 1H, ½NHC$\underline{H}_2$CH), 2.49-2.54(m, 1H, H_{6a}), 2.88 (dd, *J* = 12.2, 1H, H_{10a}), 3.14 (AB system, *J* = 14.6, 2H, NHC$\underline{H}_2$), 3.52 (s, 1H, H_9), 3.75 (s, 1H, H_{10}), 3.78 (s, 3H, OCH_3), 5.95 (s, 1H, H_7), 6.76-6.83 (m, 2H, H_2, H_4), 6.86 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.00 (d, *J* = 7.7 , 1H, H_1), 7.07 (t, *J* = 7.7, 1H, H_3), 7.19 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.5 (CH_{2cpr}), 3.6 (CH_{2cpr}), 11.3 (CH_{cpr}), 22.4 (CH_3), 28.5 (CH_3), 31.6 (C_{10a}), 40.1 (C_{6a}), 50.6 (C_9), 53.4 (C_{10}), 54.2 ($NHCH_2$), 54.8 (NH$\underline{C}H_2$CH), 55.4 (OCH_3), 77.9 (C_6), 114.2 ($C_{3'}$, $C_{5'}$), 117.3 (C_4), 119.8 (C_2), 122.1 (C_7, C_{10b}), 124.6 (C_1), 127.8 (C_3), 129.7 ($C_{2'}$, $C_{6'}$), 134.5 ($C_{1'}$), 138.3 (C_8), 155.0 (C_{4a}), 158.6 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 2.88 ppm (dd, H_{10a}) yielded NOE on 1.27 (s, CH_3), 3.75 (s, H_{10}), and 7.19 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 12.3. MS (ESI, *m/z*, %): 419.1 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{27}H_{34}N_2O_2 \cdot 2HCl$: %C 65.98, %H 7.38, %N 5.70; experimental: %C 66.74, %H 7.42, %N 5.42.

(6a*R*,9*S*,10*S*,10a*R*)-**9** (**9b**). Following general procedure B, compound **9b** was obtained from **45b** (120 mg, 0.27 mmol), as a yellow oil (104 mg, 93%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.05.

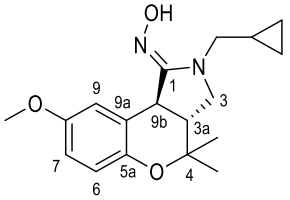


$[\alpha]^{D}_{20}$= +69.5 (c = 0.99, $CHCl_3$). Chiral HPLC (method D, t_R, min): 10.3. Elemental analysis calculated for $C_{27}H_{34}N_2O_2 \cdot 2HCl$: %C 65.98, %H 7.38, %N 5.70; experimental: %C 65.24, %H 7.23, %N 5.48. Spectroscopic data were in agreement with those described for enantiomer **9a**.

2-(Cyclopropylmethyl)-*N*-hydroxy-8-methoxy-4,4-dimethyl-2,3,3a,9b-tetrahydrochromeno[3,4-*c*]pyrrol-1(4*H*)-imine, 46

(3a*R*,9b*S*)-**46** (**46a**). **Method A:** Following general procedure B, compound **46a** was obtained from **42a** (95 mg, 0.36 mmol) and (cyclopropylmethyl)amine (62 µL, 0.72 mmol) as a yellow oil (20 mg, 17%). Chromatography: hexane to hexane/EtOAc 9:1.

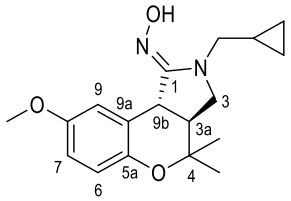
Method B: To a solution of **42a** (160 mg, 0.57 mmol) in anhydrous DCM (5 mL/mmol), (cyclopropylmethyl)amine (75 µL, 0.85 mmol) was added and the reaction was stirred at rt for 2 h. Then, the solution was evaporated under reduced pressure, and the resulting imine was dissolved in anhydrous DCM (5 mL/mmol) and DMF (0.4 mL, 5.10 mmol). After stirring for 5 min, a 1 M solution of trichlorosilane in DCM (3.58 mL, 3.58 mmol) was added dropwise and the reaction was stirred at rt overnight. Next, a sat. $NaHCO_3$ solution was added, and the mixture was extracted with EtOAc. The organic layer was washed with water (x2), and a 1:1 mixture of water/brine (x2), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography (hexane to hexane/EtOAc 9:1) to yield **46a** as an oil (144 mg, 89%, 96% *ee*).



R_f: 0.42 (hexane/EtOAc 7:3). $[\alpha]^{D}_{20}$ = -27.3 (c = 1.05, $CHCl_3$). Chiral HPLC (method H, t_R, min): 5.24. IR (ATR): ν 3295 (OH), 1660 (CNOH), 1215 (COC). ^{1}H-

NMR ($CDCl_3$): δ 0.21-0.32 (m, 2H, CH_{2cpr}), 0.47-0.57 (m, 2H, CH_{2cpr}), 1.08-1.18 (m, 1H, CH_{cpr}), 1.28 (CH_3), 1.43 (CH_3), 2.34 (ddd, *J* = 13.6, 11.0, 6.6, 1H, H_{3a}), 3.19 (dd, *J* = 10.9, 8.5, 1H, H_3), 3.31 (dd, *J* = 8.3, 6.7, 1H, H_3), 3.47-3.54 (m, 2H, ½NCH_2, H_{9b}), 3.68 (dd, *J* = 14.4, 7.1, 1H, ½NCH_2), 3.76 (s, 3H, OCH_3), 6.15 (br s, 1H, OH), 6.68-6.74 (m, 2H, H_6, H_7), 7.54-7.55 (m, 1H, H_9). ^{13}C-NMR ($CDCl_3$): δ 3.2 (CH_{2cpr}), 3.4 (CH_{2cpr}), 10.2 (CH_{cpr}), 21.5 (CH_3), 28.8 (CH_3), 40.3 (C_{9b}), 47.5 (C_{3a}), 52.0 (C_3), 54.7 (NCH_2), 55.9 (OCH_3), 76.4 (C_4), 112.3 (C_9), 114.8 (C_7), 117.4 (C_6), 121.5 (C_{9a}), 147.6 (C_{5a}), 153.0 (C_1), 153.2 (C_8). HPLC (t_R, min): 16.9. MS (ESI, *m/z*, %): 317.2 ($[M+H]^+$, 100).

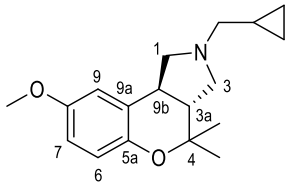
(3a*S*,9b*R*)-**46** (**46b**). Following method B, compound **46b** was obtained from **42b** (160 mg, 0.57 mmol) as an oil (129 mg, 80%, 96% *ee*).



$[\alpha]^D_{20}$= +29.3 (c = 1.08, $CHCl_3$). Chiral HPLC (method H, t_R, min): 6.14. Spectroscopic data were in agreement with those described for enantiomer **46a**.

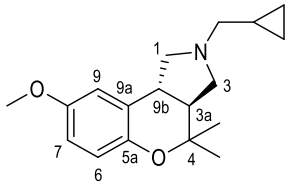
2-(Cyclopropylmethyl)-8-methoxy-4,4-dimethyl-1,2,3,3a,4,9b-hexahydro-chromeno[3,4-*c*]pyrrole, 47. To a solution of enantiomer **42a** or **42b** (1.00 eq) in anhydrous DCM (5 mL/mmol), (cyclopropylmethyl)amine (1.50 eq) was added and the reaction mixture was stirred at rt for 2 h. Then, the solvent was evaporated under reduced pressure, and the corresponding imine was dissolved in anhydrous methanol (5 mL/mmol) and cooled to 0ºC. $NaBH_4$ (2.00 eq) was then added portionwise and the reaction mixture was stirred at 0 ºC for 1 h and at rt for 1 h. Then, a sat. $NaHCO_3$ solution was added and the mixture was extracted with EtOAc (x2). The organic layers were washed with brine, dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure. The residue was purified by glass column chromatography (DCM to DCM/ethanol 95:5) to yield (3a*R*,9b*S*)- or (3a*S*,9b*R*)-**47**.

(3a*R*,9b*S*)-**47** (**47a**). Following the previous procedure, compound **47a** was obtained from **42a** (150 mg, 0.54 mmol) as an oil (47 mg, 30%, 95% *ee*).



R_f: 0.36 (DCM/ethanol 9:1). $[\alpha]^D_{20}$ = +40.5 (c = 0.75, $CHCl_3$). Chiral HPLC (method E, t_R, min): 33.5. IR (ATR): ν 1488 (CN), 1216 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.19-0.24 (m, 2H, CH_{2cpr}), 0.55-0.61 (m, 2H, CH_{2cpr}), 0.93-1.02 (m, 1H, CH_{cpr}), 1.27 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 2.11 (ddd, *J* = 12.7, 11.2, 8.2, 1H, H_{3a}), 2.58 (dd, *J* = 12.4, 6.8, 1H, ½NCH_2), 2.68 (dd, *J* = 12.4, 6.8, 1H, ½NCH_2), 2.75 (dd, *J* = 10.6, 8.9, 1H, H_1), 2.88 (app t, *J* = 10.4, 1H, H_3), 2.98 (app t, *J* = 8.9, 1H, H_3), 3.07-3.17 (m, 1H, H_{9b}), 3.64 (dd, *J* = 8.7, 6.9, 1H, H_1), 3.74 (s, 3H, OCH_3), 6.51 (d, *J* = 2.6, 1H, H_9), 6.68-6.76 (m, 2H, H_6, H_7). ^{13}C-NMR ($CDCl_3$): δ 3.9 (CH_{2cpr}), 4.2 (CH_{2cpr}), 9.8 (CH_{cpr}), 21.2 (CH_3), 29.4 (CH_3), 37.9 (C_{9b}), 49.9 (C_{3a}), 53.7 (C_3), 55.9 (OCH_3), 56.6 (C_1), 62.1 (NCH_2), 77.4 (C_4), 111.8 (C_9), 113.6 (C_7), 117.3 (C_6), 124.0 (C_{9a}), 147.8 (C_{5a}), 153.0 (C_8). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 2.11 ppm (ddd, H_{3a}) yielded NOE on 1.40 (s, CH_3); and irradiation of the signal at δ 3.07-3.17 ppm (m, H_{9b}) yielded NOE on 1.27 (s, CH_3). HPLC (t_R, min): 13.4. MS (ESI, *m/z*, %): 288.2 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{18}H_{25}NO_2 \cdot HCl$: %C 66.76, %H 8.09, %N 4.33; experimental: %C 67.11, %H 8.00, %N 4.08.

(3a*S*,9b*R*)-**47** (**47b**). Following the previous procedure, compound **47b** was obtained from **42b** (240 mg, 0.86 mmol) as an oil (81 mg, 33%, 95% *ee*).

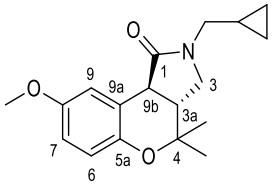


$[\alpha]^D_{20}$= -47.15 (c = 0.97, $CHCl_3$). Chiral HPLC (method E, t_R, min): 35.3. Elemental analysis calculated for $C_{18}H_{25}NO_2 \cdot HCl$: %C 66.76, %H 8.09, %N 4.33; experimental: %C 65.95, %H 7.90, %N 4.31. Spectroscopic data were in agreement with those described for enantiomer **47a**.

2-(Cyclopropylmethyl)-8-methoxy-4,4-dimethyl-2,3,3a,9b-tetrahydrochromeno[3,4-*c*]pyrrol-1(4*H*)-one, 48. To a solution of enantiomer **46a** or **46b** (1.0 eq) in methanol (21 mL/mmol) and water (50 mL/mmol), acetic acid (4.00 eq) and $NaNO_2$ (3.00 eq) were added and the mixture was stired at rt

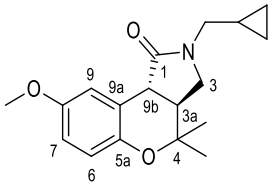
overnight. Then, the solvent was evaporated under reduced pressure and the residue was suspended in water and extracted with EtOAc (x2). The organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was purified by flash chromatography (hexane to hexane/EtOAc 85:15) to afford (3a*R*,9b*S*)- or (3a*S*,9b*R*)-**48**.

(3a*R*,9b*S*)-**48** (**48a**). Following the previous procedure, compound **48a** was obtained from **46a** (60 mg, 0.19 mmol) as an oil (22 mg, 40%, 96% *ee*).



R_f: 0.27 (hexane/EtOAc 7:3). $[\alpha]^D_{20}$ = +28.6 (c = 0.74, $CHCl_3$). Chiral HPLC (method H, t_R, min): 8.24. IR (ATR): ν 1691 (C=O), 1487 (CN), 1221 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.22-0.27 (m, 2H, CH_{2cpr}), 0.52-0.58 (m, 2H, CH_{2cpr}), 0.85-0.98 (m, 1H, CH_{cpr}), 1.32 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 2.39 (ddd, *J* = 13.8, 11.0, 6.8, 1H, H_{3a}), 3.11 (dd, *J* = 14.1, 7.2, 1H, ½NCH_2), 3.24-3.35 (m, 3H, ½NCH_2, H_3, H_{9b}), 3.43 (dd, *J* = 9.1, 6.8, 1H, H_3), 3.79 (s, 3H, OCH_3), 6.70-6.76 (m, 2H, H_6, H_7), 7.67 (dd, *J* = 2.9, 1.6, H_9). ^{13}C-NMR ($CDCl_3$): δ 3.66 (CH_{2cpr}), 3.68 (CH_{2cpr}), 9.4 (CH_{cpr}), 22.1 (CH_3), 28.5 (CH_3), 38.8 (C_{9b}), 46.7 (C_3), 46.9 (C_{3a}), 47.4 (NCH_2), 55.9 (OCH_3), 76.8 (C_4), 109.6 (C_9), 115.3 (C_7), 117.4 (C_6), 120.6 (C_{9a}), 147.5 (C_{5a}), 153.1 (C_8), 173.3 (C_1). HPLC (t_R, min): 20.8. MS (ESI, *m/z*, %): 302.2 ($[M+H]^+$, 100).

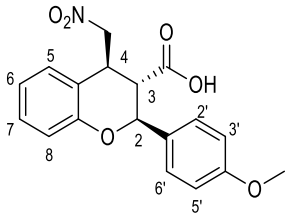
(3a*S*,9b*R*)-**48 (48b)**. Following the previous procedure, compound **48b** was obtained from **46b** (50 mg, 0.16 mmol) as an oil (12 mg, 30%, 96% *ee*).



$[\alpha]^D_{20}$= -27.1 (c = 0.17, $CHCl_3$). Chiral HPLC (method H, t_R, min): 9.84. Spectroscopic data were in agreement with those described for enantiomer **48a**.

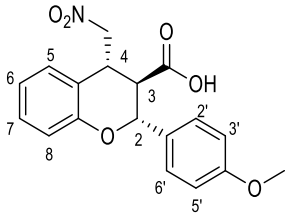
2-(4-Methoxyphenyl)-4-(nitromethyl)-3,4-dihydro-2*H*-chromene-3-carboxylic acid, 49

(2*S*,3*S*,4*S*)-**49** (**49a**). Following general procedure D, compound **49a** was obtained from **39a** (40 mg, 0.12 mmol) as a yellow solid (42 mg, quantitative).



M.p.: 112-113 ºC. R_f: 0.31 (EtAOc). $[\alpha]^{D}_{20}$ = -11.6 (c = 0.29, methanol). IR (ATR): ν 1719 (C=O), 1554 (NO_2), 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ 3.36 (t, *J* = 9.0, 1H, H_3), 3.82 (s, 3H, CH_3), 4.16-4.24 (m, 1H, H_4), 4.65 (d, *J* = 5.5, 2H, CH_2NO_2), 5.12 (d, *J* = 9.0, 1H, H_2), 6.92 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 6.94-7.04 (m, 2H, H_6, H_8), 7.17-7.24 (m, 2H, H_5, H_7), 7.34 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 37.1 (C_4), 49.6 (C_3), 55.5 (CH_3), 77.6 (CH_2NO_2), 78.2 (C_2), 114.3 ($C_{3'}$, $C_{5'}$), 118.1 (C_8), 119.3 (C_{4a}), 122.2 (C_6), 127.2 (C_5), 128.5 ($C_{2'}$, $C_{6'}$), 129.2 (C_7), 129.6 ($C_{1'}$), 154.9 (C_{8a}), 160.2 ($C_{4'}$), 174.9 (CO_2H). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.16-4.24 ppm (m, H_4) yielded NOE on 5.12 (d, H_2); and irradiation of the signal at δ 4.65 ppm (d, CH_2NO_2) yielded NOE on 3.36 (t, H_3). HPLC (t_R, min): 18.44. MS (ESI, *m/z*, %): 344.1 ($[M+H]^+$, 100).

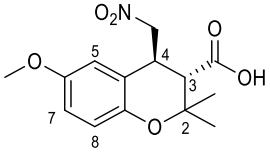
(2*R*,3*R*,4*R*)-**49** (**49b**). Following general procedure D, compound **49b** was obtained from **39b** (65 mg, 0.20 mmol) as a yellow solid (59 mg, 87%).



$[\alpha]^{D}_{20}$= +10.4 (c = 0.33, methanol). Spectroscopic data were in agreement with those described for enantiomer **49a**.

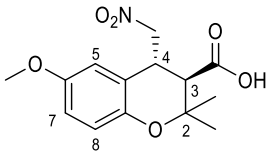
6-Methoxy-2,2-dimethyl-4-(nitromethyl)-3,4-dihydro-2*H*-chromene-3-carboxylic acid, 50

(3*S*,4*S*)-**50** (**50a**). Following general procedure D, compound **50a** was obtained from **42a** (270 mg, 0.97 mmol) as a white solid (247 mg, 86%).



M.p.: 118-120 ºC. R_f: 0.29 (AcOEt). $[\alpha]^D_{20}$ = -8.3 (c = 0.28, $CHCl_3$). IR (ATR): ν 2925 (OH), 1709 (C=O), 1553 (NO_2), 1243 (COC). ^{1}H-NMR ($CDCl_3$): δ 1.26 (CH_3), 1.58 (CH_3), 3.10 (dd, *J* = 11.2, 1H, H_3), 3.76 (s, 3H, OCH_3), 3.92-3.99 (m, 1H, H_4), 4.70 (dd, *J* = 13.5, 4.4, 1H, ½CH_2NO_2), 4.77 (dd, *J* = 13.5, 5.6, 1H, ½CH_2NO_2), 6.71-6.74 (m, 1H, H_5) 6.77-6.81 (m, 2H, H_7, H_8). ^{13}C-NMR ($CDCl_3$): δ 20.6 (CH_3), 28.7 (CH_3), 35.0 (C_4), 52.2 (C_3), 55.9 (OCH_3), 75.2 (C_2), 78.3 (CH_2NO_2), 111.6 (C_5), 115.2 (C_7), 119.2 (C_8), 119.7 (C_{4a}), 146.8 (C_{8a}), 154.2 (C_6), 176.5 (COOH). HPLC (t_R, min): 16.8. MS (ESI, *m/z*, %): 294.1 ([M-H]$^-$, 20).

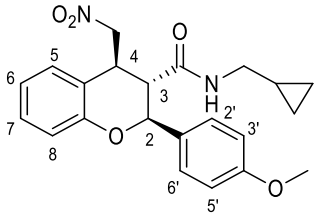
(3*R*,4*R*)-**50** (**50b**). Following general procedure D, compound **50b** was obtained from **42b** (364 mg, 1.29 mmol) as a white solid (300 mg, 79%).



$[\alpha]^D_{20}$= +6.5 (c = 0.18, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **50a**.

N-(Cyclopropylmethyl)-2-(4-methoxyphenyl)-4-(nitromethyl)-3,4-dihydro-2*H*-chromene-3-carboxamide, 51

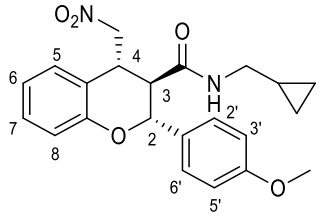
(2*S*,3*S*,4*S*)-**51** (**51a**). Following general procedure E, compound **51a** was obtained from **49a** (45 mg, 0.13 mmol) as an oil (30 mg, 58%).



R_f: 0.53 (EtAOc). $[\alpha]^D_{20}$ = -18.0 (c = 0.48, $CHCl_3$). IR (ATR): ν 3316 (NH), 1650 (C=O), 1553 (NO_2), 1243 (COC). ^{1}H-NMR ($CDCl_3$): δ -0.16-0.02 (m, 2H, CH_{2cpr}), 0.26-0.34 (m, 2H, CH_{2cpr}), 0.47-0.60 (m, 1H, CH_{cpr}), 2.80-2.88 (m, 2H, NHC$\underline{H}_2$), 2.99 (t, *J* = 10.2, 1H, H_3), 3.81 (s, 3H, CH_3), 4.17-4.25 (m, 1H, H_4), 4.65 (dd, *J* = 13.1, 3.5, 1H, ½CH_2NO_2), 4.99 (d, *J* = 10.2, 1H, H_2), 5.00 (dd, *J* = 13.1, 4.8, 1H, ½CH_2NO_2), 5.33-5.41 (m, 1H, NH), 6.91 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 6.93 (d, *J* = 7.9, 1H, H_8), 6.99 (td, *J* = 7.6, 1.2, 1H, H_6), 7.20 (t, *J* = 7.8, 1H, H_7), 7.30 (d, *J* = 7.8, 1H, H_5), 7.34 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.3 (CH_{2cpr}), 3.5 (CH_{2cpr}), 10.4 (CH_{cpr}), 37.8 (C_4), 44.6 ($NHCH_2$), 51.7 (C_3), 55.5 (CH_3), 76.0 (CH_2NO_2), 79.5

(C_2), 114.3 ($C_{3'}$, $C_{5'}$), 117.9 (C_8), 119.2 (C_{4a}), 121.6 (C_6), 126.6 (C_5), 128.3 ($C_{2'}$, $C_{6'}$), 128.9 (C_7), 130.7 ($C_{1'}$), 155.2 (C_{8a}), 160.2 ($C_{4'}$), 169.8 (CONH). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 2.99 ppm (t, H_3) yielded NOE on 7.34 (d, $H_{2'}$, $H_{6'}$); irradiation of the signal at δ 4.17-4.15 ppm (m, H_4) yielded NOE on 4.99 (d, H_2); and irradiation of the signal at δ 4.65 ppm (d, ½CH_2NO_2) yielded NOE on 2.99 (t, H_3). HPLC (t_R, min): 19.97. MS (ESI, *m/z*, %): 397.2 ([M+H]$^+$, 100).

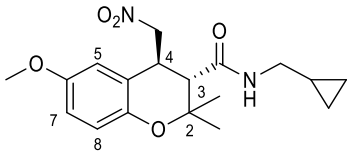
(2*R*,3*R*,4*R*)-**51** (**51b**). Following general procedure E, compound **51b** was obtained from **49b** (60 mg, 0.17 mmol) as an oil (38 mg, 55%).



[α]$^D_{20}$= +20.8 (c = 0.47, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **51a**.

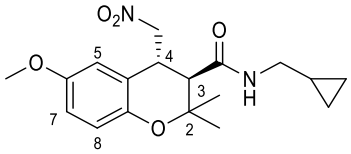
N-(Cyclopropylmethyl)-6-methoxy-2,2-dimethyl-4-(nitromethyl)-3,4-dihydro-2*H*-chromene-3-carboxamide, 52

(3*S*,4*S*)-**52 (52a).** Following general procedure E, compound **52a** was obtained from **50a** (170 mg, 0.97 mmol) as an oil (179 mg, 90%).



R_f: 0.32 (hexane/AcOEt 7:3). [α]$^D_{20}$ = +18.9 (c = 1.00, $CHCl_3$). IR (ATR): ν 3320 (NH), 1648 (C=O), 1549 (NO_2), 1225 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.21-0.26 (m, 2H, CH_{2cpr}), 0.52-0.55 (m, 2H, CH_{2cpr}), 0.97-1.03 (m, 1H, CH_{cpr}), 1.27 (CH_3), 1.48 (CH_3), 2.82 (d, *J* = 11.7, 1H, H_3), 3.10-3.27 (m, 2H, NHC$\underline{H}_2$), 3.76 (s, 3H, OCH_3), 3.85 (app dt, *J* = 11.7, 3.9, 1H, H_4), 4.57 (dd, *J* = 13.4, 3.3, 1H, ½CH_2NO_2), 4.97 (dd, *J* = 13.4, 4.6, 1H, ½CH_2NO_2), 6.12 (br s, 1H, NH), 6.74-6.77 (m, 3H, H_5, H_7, H_8). ^{13}C-NMR ($CDCl_3$): δ 3.60 (CH_{2cpr}), 3.65 (CH_{2cpr}), 11.0 (CH_{cpr}), 20.1 (CH_3), 28.7 (CH_3), 35.5 (C_4), 44.8 ($NHCH_2$), 52.6 (C_3), 55.9 (OCH_3), 75.5 (C_2), 76.2 (CH_2NO_2), 111.5 (C_5), 114.9 (C_7), 118.9 (C_8), 119.4 (C_{4a}), 147.3 (C_{8a}), 153.7 (C_6), 170.6 (NHCO). HPLC (t_R, min): 19.4. MS (ESI, *m/z*, %): 349.2 ([M+H]$^+$, 100).

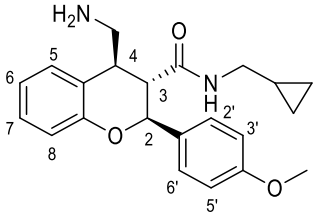
(3*R*,4*R*)-**52** (**52b**). Following general procedure E, compound **52b** was obtained from **50b** (63 mg, 1.29 mmol) as an oil (74 mg, quantitative).



$[\alpha]^{D}_{20}$= -16.9 (c = 1.05, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **52a**.

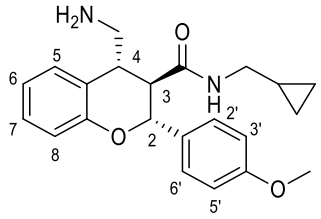
4-(Aminomethyl)-*N*-(cyclopropylmethyl)-2-(4-methoxyphenyl)-3,4-dihydro-2*H*-chromene-3-carboxamide, 5

(2*S*,3*S*,4*S*)-**5** (**5a**). Following general procedure C, compound **5a** was obtained from **51a** (80 mg, 0.20 mmol) as white solid (47 mg, 64%, 94% *ee*). Chromatography: DCM to DCM/methanol 98:2.



M.p.: 129-130 ºC. R_f: 0.52 (DCM/methanol 9:1). $[\alpha]^{D}_{20}$ = -5.6 (c = 0.44, methanol). Chiral HPLC (method H, t_R, min): 8.00. IR (ATR): ν 3309 (NH), 1641 (C=O), 1241 (COC). ^{1}H-NMR (methanol-*d4*): δ -0.10-0.00 (m, 2H, CH_{2cpr}), 0.24-0.34 (m, 2H, CH_{2cpr}), 0.52-0.66 (m, 1H, CH_{cpr}), 2.72 (dd, *J* = 13.8, 7.2, 1H, ½NHC$\underline{H}_2$), 2.83-2.97 (m, 2H, H_3, ½$NH_2C\underline{H}_2$), 2.89 (dd, *J* = 13.8, 3.5, 1H, ½NHC$\underline{H}_2$), 3.31-3.37 (m, 1H, ½$NH_2C\underline{H}_2$), 3.53 (dt, *J* = 11.4, 3.5, 1H, H_4), 3.80 (s, 3H, CH_3), 4.88 (d, *J* = 10.1, 1H, H_2), 6.87 (dd, *J* = 8.1, 1.2, 1H, H_8), 6.93 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.00 (td, *J* = 7.6, 1.3, 1H, H_6), 7.16 (t, *J* = 7.3, 1H, H_7), 7.32-7.40 (m, 1H, H_5), 7.35 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR (methanol-*d4*): δ 3.6 (CH_{2cpr}), 3.8 (CH_{2cpr}), 11.2 (CH_{cpr}), 41.5 (C_4), 42.9 (NH_2CH_2), 44.9 ($NHCH_2$), 51.5 (C_3), 55.8 (CH_3), 80.6 (C_2), 114.8 ($C_{3'}$, $C_{5'}$), 118.4 (C_8), 122.6 (C_6), 123.2 (C_{4a}), 128.2 (C_5), 128.8 (C_7), 129.8 ($C_{2'}$, $C_{6'}$), 132.3 ($C_{1'}$), 157.5 (C_{8a}), 161.4 ($C_{4'}$), 173.2 (NHCO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 4.88 (d, H_2) yielded NOE on 3.53 (dt, H_4). HPLC (t_R, min): 13.48. MS (ESI, *m/z*, %): 367.2 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{22}H_{26}N_2O_3 \cdot HCl$: %C 65.58, %H 6.75, %N 6.95; experimental: %C 63.78, %H 6.80, %N 6.64.

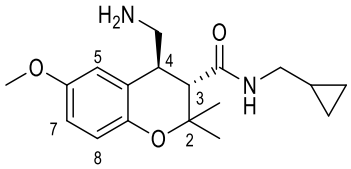
(2*R*,3*R*,4*R*)-**5** (**5b**). Following general procedure C, compound **5b** was obtained from **51b** (72 mg, 0.18 mmol) as a white solid (44 mg, 67%, 94% *ee*).



$[\alpha]^{D}_{20}$= +5.3 (c = 0.47, methanol). Chiral HPLC (method H, t_R, min): 7.14. Elemental analysis calculated for $C_{22}H_{26}N_2O_3 \cdot HCl$: %C 65.58, %H 6.75, %N 6.95; experimental: %C 64.60, %H 6.56, %N 6.74. Spectroscopic data were in agreement with those described for enantiomer **5a**.

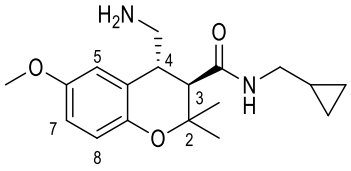
4-(Aminomethyl)-*N*-(cyclopropylmethyl)-6-methoxy-2,2-dimethyl-3,4-dihydro-2*H*-chromene-3-carboxamide, 6

(3*S*,4*S*)-**6** (**6a**). Following general procedure C, compound **6a** was obtained from **52a** (179 mg, 0.52 mmol) as an oil (144 mg, 87%, 96% *ee*). Chromatography: DCM to DCM/ethanol 9:1.



R_f: 0.36 (DCM/ethanol 9:1). $[\alpha]^{D}_{20}$ = +6.7 (c = 0.83, $CHCl_3$). Chiral HPLC (method B, t_R, min): 7.3. IR (ATR): ν 3309 (NH), 1651 (CO), 1254 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.20-0.25 (m, 2H, CH_{2cpr}), 0.50-0.53 (m, 2H, CH_{2cpr}), 0.94-1.02 (m, 1H, CH_{cpr}), 1.30 (CH_3), 1.46 (CH_3), 2.79-2.86 (m, 2H, H_3, ½$NH_2C\underline{H}_2$), 3.08-3.24 (m, 2H, $NHC\underline{H}_2$), 3.31-3.41 (m, 2H, H_4, ½$NH_2C\underline{H}_2$), 3.77 (s, 3H, OCH_3), 6.44 (br s, 1H, NHCO), 6.69-6.77 (m, 2H, H_7, H_8), 6.80 (d, *J* = 2.5, 1H, H_5). ^{13}C-NMR ($CDCl_3$): δ 3.60 (CH_{2cpr}), 3.65 (CH_{2cpr}), 11.0 (CH_{cpr}), 20.5 (CH_3), 28.6 (CH_3), 36.7 (C_4), 42.2 (NH_2CH_2), 44.5 ($NHCH_2$), 51.6 (C_3), 55.9 (OCH_3), 75.4 (C_2), 111.9 (C_5), 113.5 (C_7), 118.6 (C_8), 122.7 (C_{4a}), 148.4 (C_{8a}), 153.9 (C_6), 172.1 (NHCO). HPLC (t_R, min): 12.8. MS (ESI, *m/z*, %): 319.2 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{18}H_{26}N_2O_3 \cdot HCl$: %C 60.92, %H 7.67, %N 7.89; experimental: %C 60.96, %H 7.46, %N 7.48.

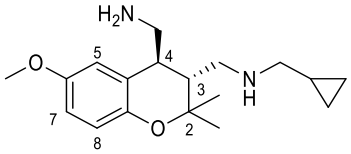
(3*R*,4*R*)-**6** (**6b**). Following general procedure C, compound **6b** was obtained from **52b** (240 mg, 0.69 mmol) as an oil (142 mg, 65%, 96% *ee*). Chromatography: DCM to DCM/ethanol 9:1.



$[\alpha]^{D}_{20}$= -8.8 (c = 0.97, $CHCl_3$). Chiral HPLC (method B, t_R, min): 8.7. Elemental analysis calculated for $C_{18}H_{26}N_2O_3 \cdot HCl$: %C 60.92, %H 7.67, %N 7.89; experimental: %C 60.66, %H 7.56, %N 7.54. Spectroscopic data were in agreement with those described for enantiomer **6a**.

1-[4-(Aminomethyl)-6-methoxy-2,2-dimethyl-3,4-dihydro-2*H*-chromen-3-yl]-*N*-(cyclopropylmethyl)methanamine, 7. To a solution of enantiomer **6a** or **6b** (1.00 eq) in anhydrous THF (5 mL/mmol), $LiAlH_4$ (5.00 eq) was added portionwise at 0 ºC and the mixture was refluxed for 24 h. The reaction was then quenched with water and 10% aqueous NaOH and stirred for 10 min. Then, the mixture was extracted with EtOAc (x2) and the combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by glass column cromatography (DCM to DCM/ethanol/NH_3 8:2:0.05) to afford compound (3*R*,4*S*)- or (3*S*,4*R*)-**7**.

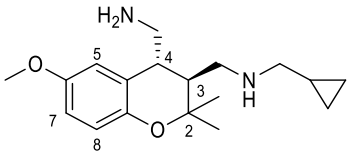
(3*R*,4*S*)-**7** (**7a**). Following previous procedure, compound **7a** was obtained from **6a** (55 mg, 0.17 mmol) as an oil (10 mg, 20%, 96% *ee*).



R_f: 0.27 (DCM/ethanol/NH_3) 9:1:0.05). $[\alpha]^{D}_{20}$ = -10.56 (c = 0.18, $CHCl_3$). Chiral HPLC (method I, t_R, min): 4.36. ^{1}H-NMR ($CDCl_3$): δ 0.10-0.15 (m, 2H, CH_{2cpr}), 0.46-0.52 (m, 2H, CH_{2cpr}), 0.85-1.01 (m, 1H, CH_{cpr}), 1.09 (CH_3), 1.49 (CH_3), 1.93 (ddd, *J* = 9.5, 5.8, 4.0, 1H, H_3), 2.48 (d, *J* = 6.8, 2H, NHC$\underline{H}_2$CH$_{cpr}$), 2.56 (dd, *J* = 12.4, 5.9, 1H, ½NHC$\underline{H}_2$), 2.66-2.72 (m, 1H, H_4), 2.81 (dd, *J* = 12.4, 3.8, 1H, ½NHC$\underline{H}_2$), 3.16 (dd, *J* = 13.4, 3.4, 1H, ½NH$_2$C$\underline{H}_2$), 3.23 (dd, *J* = 13.4, 4.5, 1H, ½NH$_2$C$\underline{H}_2$), 3.76 (s, 3H, OCH_3), 6.66-6.75 (m, 2H, H_7, H_8), 6.78 (d, *J* = 2.6, 1H, H_5). ^{13}C-NMR ($CDCl_3$): δ 3.56 (CH_{2cpr}), 3.59 (CH_{2cpr}), 11.2 (CH_{cpr}), 21.1 (CH_3), 28.7 (CH_3), 41.2

(C_4), 44.4 (C_3), 44.5 (NH_2CH_2), 51.9 ($NHCH_2$), 55.4 ($NH\underline{C}H_2CH_{cpr}$), 55.8 ($OCH_3$), 76.9 ($C_2$), 112.5 ($C_5$), 113.2 ($C_7$), 118.4 ($C_8$), 125.2 ($C_{4a}$), 148.6 ($C_{8a}$), 153.8 ($C_6$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 1.09 ppm (s, CH_3) yielded NOE on 2.66-2.72 (m, H_4), and 2.81 (dd, ½$NHC\underline{H}_2$); and irradiation of the signal at δ 1.49 ppm (m, CH_3) yielded NOE on 1.93 (ddd, H_3). HPLC (t_R, min): 4.10. MS (ESI, *m/z*, %): 305.2 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{18}H_{28}N_2O_2 \cdot 2HCl$: %C 57.29, %H 8.01, %N 7.42; experimental: %C 57.89, %H 7.67, %N 7.12.

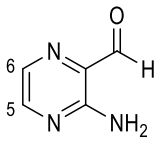
(3*S*,4*R*)-**7** (**7b**). Following previous procedure, compound **7b** was obtained from **6b** (72 mg, 0.23 mmol) as an oil (15 mg, 22%, 96% *ee*).



$[\alpha]^D_{20}$= +9.05 (c = 0.11, $CHCl_3$). Chiral HPLC (method I, t_R, min): 4.77. Elemental analysis calculated for $C_{18}H_{28}N_2O_2 \cdot 2HCl$: %C 57.29, %H 8.01, %N 7.42; experimental: %C 57.79, %H 7.81, %N 7.48. Spectroscopic data were in agreement with those described for enantiomer **7a**.

*4.1.5. Synthesis of pyridopyrazine scaffold. Final compounds **10**, **55** and **57***

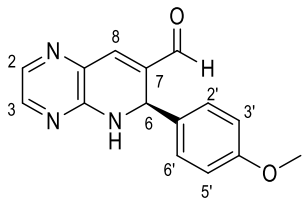
3-Aminopyrazine-2-carbaldehyde, 53. To a solution of methyl 3-amino-2-pyrazinecarboxylate (1.00 g, 6.53 mmol) in anhydrous THF (13 mL) at -78 ºC, DIBALH (23 mL, 1 M in THF) was added dropwise and the reaction was stirred at this temperature for 4 h. After that time, the reaction was quenched with methanol, and 1 M HCl and a sat. Rochelle salt solution were added. After stirring at rt for 1 h, the mixture was extracted with EtOAc (x3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography (hexane to hexane/EtOAc 7:3) to afford compound **53** as a yellow solid (420 mg, 52%). The spectroscopic data were consistent with those previously reported.[73]



M.p.: 118-120 ºC (lit.[73] 118.9ºC). R_f: 0.35 (hexane/EtOAc 1:1). ^{1}H-NMR ($CDCl_3$): δ 8.05 (d, *J* = 2.2 Hz, 1H, H_6), 8.21 (d, *J* = 2.1 Hz, 1H, H_5), 10.02 (s, 1H, CHO).

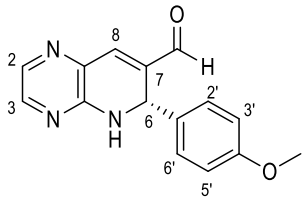
6-(4-Methoxyphenyl)-5,6-dihydropyrido[2,3-*b*]pyrazine-7-carbaldehyde, 54. To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq), *p*-methoxycinnamaldehyde (1.50 eq), and acetic acid (0.20 eq) in anhydrous chloroform (1mL/mmol), **53** (1.00 eq) was added and the reaction was stirred at rt for 7 days. Next, the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (hexane to hexane/EtOAc 1:1) affording intermediate (*R*)- or (*S*)-**54**.

(*R*)-**54** (**54a**). Following the previous procedure, compound **54a** was obtained from **53** (50 mg, 0.41 mmol), using the *S* enantiomer of the catalyst (26 mg, 0.08 mmol), as a yellow oil (44 mg, 40%).



R_f: 0.25 (hexane/EtOAc 4:6). $[\alpha]^D_{20}$ = -255.3 (c = 0.53, $CHCl_3$). IR (ATR): ν 3236 (NH), 1671 (C=O), 1246 (COC). ^{1}H-NMR ($CDCl_3$): δ 3.76 (s, 3H, CH_3), 5.80 (s, 1H, H_6), 5.97 (br s, 1H, NH), 6.82 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.26 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 7.32 (s, 1H, H_8), 7.80 (d, *J* = 2.7, 1H, H_3), 7.85 (d, *J* = 2.6, 1H, H_2), 9.56 (s, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 54.5 (C_6), 55.4 (CH_3), 114.3 ($C_{3'}$, $C_{5'}$), 127.8 ($C_{2'}$, $C_{6'}$), 133.4 (C_{8a}), 135.0 (C_2), 135.4 ($C_{1'}$), 138.4 (C_7), 140.8 (C_8), 144.8 (C_3), 153.2 (C_{4a}), 159.8 ($C_{4'}$), 190.4 (CHO).

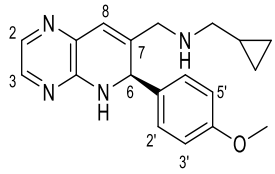
(*S*)-**54** (**54b**). Following the previous procedure, compound **54b** was obtained from **53** (50 mg, 0.41 mmol), using the *R* enantiomer of the catalyst (26 mg, 0.08 mmol), as a yellow oil (49 mg, 45%).



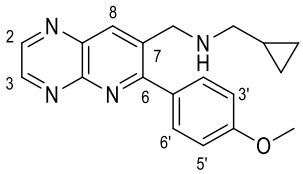
$[\alpha]^{D}_{20}$= +260.9 (c = 0.55, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **54a**.

1-Cyclopropyl-*N*-{[(6*S*)-6-(4-methoxyphenyl)-5,6-dihydropyrido[2,3-*b*]pyrazin-7-yl]methyl}methanamine, (*R*)-10 and 1-cyclopropyl-*N*-{[6-(4-methoxyphenyl)pyrido[2,3-*b*]pyrazin-7-yl]methyl}methanamine, 55

Following general procedure B, compounds (*R*)-**10** and **55** were obtained from **54a** (140 mg, 0.52 mmol) and (cyclopropylmethyl)amine (90 µL, 1.04 mmol) as oils in 80% (138 mg) and 10% (16 mg) yields, respectively. Chromatography: DCM to DCM/ethanol 9:1.



(*R*)-**10**: R_f: 0.17 (DCM/ethanol/NH_3 9:1:0.05). $[\alpha]^{D}_{20}$ = +195.3 (c = 0.40, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 0.05-0.10 (m, 2H, CH_{2cpr}), 0.42-0.48 (m, 2H, CH_{2cpr}), 0.85-0.96 (m, 1H, CH_{cpr}), 2.38 (dd, *J* = 12.1, 6.9, 1H, ½NHC$\underline{H}_2$CH), 2.46 (dd, *J* = 12.1, 6.9, 1H, ½NHC$\underline{H}_2$CH), 3.12 (s, 2H, NHC$\underline{H}_2$), 3.79 (s, 3H, CH_3), 5.02 (s, 1H, NH), 5.56 (s, 1H, H_6), 6.58 (s, 1H, H_8), 6.86 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.29 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$) 7.63 (d, *J* = 2.9, 1H, H_2), 7.66 (d, *J* = 2.9, 1H, H_3). ^{13}C-NMR ($CDCl_3$): δ 3.5 ($2CH_{2cpr}$), 10.5 (CH_{cpr}), 51.6 ($NHCH_2$), 54.2 (NHC$\underline{H}_2$CH), 55.3 (CH_3), 59.6 (C_6), 114.4 ($C_{3'}$, $C_{5'}$), 122.4 (C_8), 128.1 ($C_{2'}$, $C_{6'}$), 133.2 (C_3), 134.8 (C_1), 136.5 (C_{8a}), 140.8 (C_2), 142.8 (C_7), 150.8 (C_{4a}), 160.0 ($C_{4'}$). This compound is unstable and evolves at rt to derivative **55**.

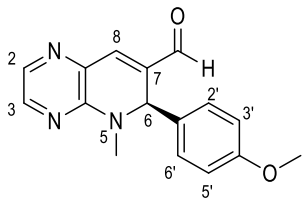


55: R_f: 0.20 (DCM/ethanol/NH_3 9:1:0.05). ^{1}H-NMR ($CDCl_3$): δ 0.03-0.08 (m, 2H, CH_{2cpr}), 0.42-0.48 (m, 2H, CH_{2cpr}), 0.88-0.98 (m, 1H, CH_{cpr}), 2.49 (d, *J* = 6.8 Hz, 2H, NHC$\underline{H}_2$CH), 3.89 (s, 3H, CH_3), 4.10 (s, 2H, NHC$\underline{H}_2$), 7.03 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.76 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 8.61 (s, 1H, H_8), 8.90 (d, *J* = 1.7, 1H, H_2), 9.02 (d, *J* = 1.7, 1H, H_3). ^{13}C-NMR ($CDCl_3$): δ 3.5 ($2CH_{2cpr}$), 11.3 (CH_{cpr}), 51.2 ($NHCH_2$), 54.6 (NH$\underline{C}H_2$CH), 55.5 (CH_3), 113.9 ($C_{3'}$, $C_{5'}$), 131.0 ($C_{2'}$, $C_{6'}$), 131.6 ($C_{1'}$),

137.4, 137.6 (C_7, C_{8a}), 138.2 (C_8), 145.8 (C_2), 147.6 (C_3), 150.1 (C_{4a}), 160.7 ($C_{4'}$), 163.5 (C_6). Elemental analysis calculated for $C_{18}H_{23}N_4O_2 \cdot HCl$: %C 60.88, %H 6.80, %N 14.95; experimental: %C 59.29, %H 6.65, %N 13.94.

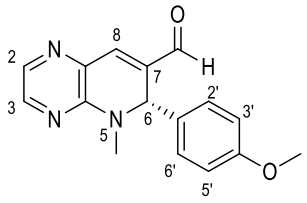
6-(4-Methoxyphenyl)-5-methyl-5,6-dihydropyrido[2,3-*b*]pyrazine-7-carbaldehyde, 56. To a solution of enantiomer **54a** or **54b** (1.00 eq) in anhydrous DMF (5 mL/mmol), Cs_2CO_3 (2.20 eq) and iodomethane (5.00 eq) were added and the reaction was stirred for 1 h. Then, water was added and the mixture was neutralized with 1 M HCl and extracted with EtOAc (x3). The combined organic layers were washed with a 1:1 mixture of water/brine (x4), dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford (*R*)- or (*S*)-**56**, which was used in the next step without further purification.

(*R*)-**56** (**56a**). Following the previous procedure, compound **56a** was from **54a** (65 mg, 0.24) as a yellow oil (64 mg, 95%).



R_f: 0.40 (hexane/EtOAc 1:1). $[\alpha]^D_{20}$ = -228.7 (c = 0.60, $CHCl_3$). IR (ATR): ν 1675 (C=O), 1250 (COC). ^{1}H-NMR ($CDCl_3$): δ 2.95 (s, 3H, NCH_3), 3.76 (s, 3H, OCH_3), 5.63 (s, 1H, H_6), 6.81 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.22 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 7.30 (s, 1H, H_8), 7.81 (d, *J* = 2.6, 1H, H_2), 7.97 (d, *J* = 2.6, 1H, H_3), 9.54 (s, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 33.5 (NCH_3), 55.4 (OCH_3), 61.8 (C_6), 114.2 ($C_{3'}$, $C_{5'}$), 128.0 ($C_{2'}$, $C_{6'}$), 132.5 ($C_{1'}$), 133.4 (C_2), 134.5 (C_{8a}), 137.8 (C_7), 140.5 (C_8), 144.9 (C_3), 153.5 (C_{4a}), 159.9 ($C_{4'}$), 190.3 (CHO). HPLC (t_R, min): 17.7. MS (ESI, *m/z*, %): 282.1 ($[M+H]^+$, 100).

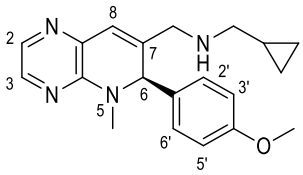
(*S*)-**56** (**56b**). Following the previous procedure, compound **56b** was from **54b** (180 mg, 0.67) as a yellow oil (188 mg, quantitative).



$[\alpha]^{D}_{20}$= +232.2 (c = 0.50, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **56a**.

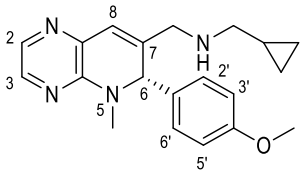
1-Cyclopropyl-*N*-{[-6-(*p*-methoxyphenyl)-5-methyl-5,6-dihydropyrido-[2,3-*b*]pyrazin-7-yl]methyl}methanamine, 57

(*R*)-**57** (**57a**). Following general procedure B, compound **57a** was obtained from **56a** (65 mg, 0.23 mmol) and (cyclopropylmethyl)amine (40 µL, 0.46 mmol) as a yellow oil (35 mg, 50%, 98% *ee*). Chromatography: DCM to DCM/ethanol 9:1.



R_f: 0.40 (DCM/ethanol 9:1). $[\alpha]^{D}_{20}$ = -217.8 (c = 1.00, $CHCl_3$). Chiral HPLC (method E, t_R, min): 12.43. IR (ATR): ν 3405 (NH), 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.04-0.09 (m, 2H, CH_{2cpr}), 0.42-0.48 (m, 2H, CH_{2cpr}), 0.82-0.95 (m, 1H, CH_{cpr}), 2.36 (dd, *J* = 12.1, 6.9, 1H, ½NHC$\underline{H}_2$CH), 2.45 (dd, *J* = 12.1, 6.8, 1H, ½NHC$\underline{H}_2$CH), 2.83 (s, 3H, NCH_3), 3.12 (s, 2H, NHC$\underline{H}_2$), 3.79 (s, 3H, OCH_3), 5.31 (s, 1H, H_6), 6.54 (s, 1H, H_8), 6.82-6.85 (m, 2H, $H_{3'}$, $H_{5'}$), 7.17-7.22 (m, 2H, $H_{2'}$, $H_{6'}$), 7.60 (d, *J* = 3.0, 1H, H_2), 7.75 (d, *J* = 2.9 Hz, 1H, H_3). ^{13}C-NMR ($CDCl_3$): δ 3.4 (CH_{2cpr}), 3.5 (CH_{2cpr}), 11.3 (CH_{cpr}), 33.3 (NCH_3), 51.7 ($NHCH_2$), 54.4 (NH$\underline{C}H_2$CH), 55.4 (OCH_3), 66.8 (C_6), 114.3 ($C_{3'}$, $C_{5'}$), 122.2 (C_8), 128.2 ($C_{2'}$, $C_{6'}$), 131.4 (C_2), 132.6 ($C_{1'}$), 137.6 (C_{8a}), 140.6 (C_3), 143.2 (C_7), 151.4 (C_{4a}), 159.9 ($C_{4'}$). HPLC (t_R, min): 12.9. MS (ESI, *m/z*, %): 266.1 ([M-NHCH$_2$CH(CH$_2$)$_2$]$^+$, 100), 337.2 ([M+H]$^+$, 80). Elemental analysis calculated for $C_{20}H_{24}N_4O\cdot 2HCl\cdot 2H_2O$: %C 53.94, %H 6.79, %N 12.58; experimental: %C 54.07, %H 6.71, %N 12.75.

(*S*)-**57** (**57b**). Following general procedure B, compound **57b** was obtained from **56b** (180 mg, 0.64 mmol), as a yellow oil (105 mg, 49%, 98% *ee*). Chromatography: DCM to DCM/ethanol 9:1.

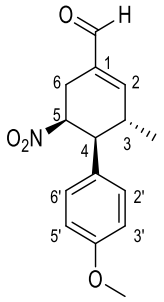


$[\alpha]^{D}_{20}$= +220.1 (c = 0.90, $CHCl_3$). Chiral HPLC (method E, t_R, min): 13.12. Elemental analysis calculated for $C_{20}H_{24}N_4O \cdot 2HCl \cdot 2H_2O$: %C 53.94, %H 6.79, %N 12.58; experimental: %C 53.59, %H 6.91, %N 12.16. Spectroscopic data were in agreement with those described for enantiomer **57a**.

*4.1.6. Synthesis of tetrasubstituted cyclohexene scaffold. Final compounds **11**-**20***

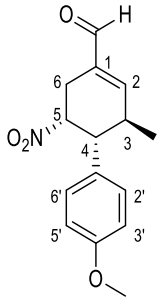
4-(4-Methoxyphenyl)-3-methyl-5-nitrocyclohex-1-ene-1-carbaldehyde, 58. To a solution of (*S)*- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.20 eq) and *trans-p*-methoxy-*β*-nitrostyrene (1.00 eq) in anhydrous toluene (0.8 mL/mmol) at 0 ºC, propionaldehyde (1.20 eq) and acrylaldehyde (1.05 eq) were added successively. After stirring at 0 ºC for 1 h, the reaction was warmed up to rt and stirred overnight. Then, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (hexane to hexane/EtOAc 85:15) to afford title compound (3*S*,4*S*,5*S*)- or (3*R*,4*R*,5*R*)-**58**.

(3*S*,4*S,5S*)-**58** (**58a**). Following the previous procedure, compound **58a** was obtained from *trans-p*-methoxy-*β*-nitrostyrene (351 mg, 1.96 mmol), using the *S* enantiomer of the catalyst (127 mg, 0.39 mmol), as a yellow solid (321 mg, 59%).



M.p.: 126-128 ºC. R_f: 0.33 (hexane/EtOAc 7:3). $[\alpha]^{D}_{20}$ = +13.3 (c = 0.97, $CHCl_3$). IR (ATR): ν 1681 (C=O), 1546 (NO_2), 1251 (COC). ^{1}H-NMR ($CDCl_3$, 700 MHz): δ 1.22 (d, *J* = 7.2 , 3H, CH_3), 2.80 (app dt, *J* = 5.4, 1.7, 2H, $2H_6$), 3.12 (dd, *J* = 6.9, 3.8, 1H, H_4), 3.16-3.21 (m, 1H, H_3), 3.79 (s, 3H, OCH_3), 4.90 (td, *J* = 5.6, 3.8, 1H, H_5), 6.84 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 6.91 (dt, *J* = 3.2, 1.7, 1H, H_2), 7.00 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 9.57 (s, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 19.9 (CH_3), 24.6 (C_6), 34.4 (C_3), 48.9 (C_4), 55.4 (OCH_3), 83.9 (C_5), 114.5 ($C_{3'}$, $C_{5'}$), 128.8 ($C_{2'}$, $C_{6'}$), 129.5 ($C_{1'}$), 136.0 (C_1), 154.2 (C_2), 159.5 ($C_{4'}$), 192.6 (CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 3.16-3.21 ppm (m, H_3) yielded NOE on 7.00 (d, $H_{2'}$, $H_{6'}$); and irradiation of the signal at δ 4.90 ppm (td, H_5) yielded NOE on 3.12 (dd, H_4). HPLC (t_R, min): 12.9. MS (ESI, *m/z*, %): 274.1 ([M-H]$^-$, 100).

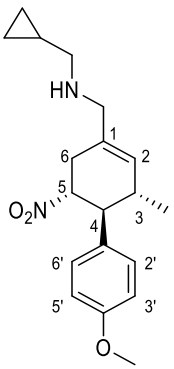
(3*R*,4*R*,5*R*)-**58** (**58b**). Following the previous procedure, compound **58b** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (550 mg, 3.07 mmol), using the *R* enantiomer of the catalyst (200 mg, 0.06 mmol), as a yellow solid (423 mg, 50%).



$[\alpha]^{D}_{20}$= -12.9 (c = 1.10, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **58a**.

1-Cyclopropyl-*N*-{[4-(4-methoxyphenyl)-3-methyl-5-nitrocyclohex-1-en-1-yl]methyl} methanamine, 59

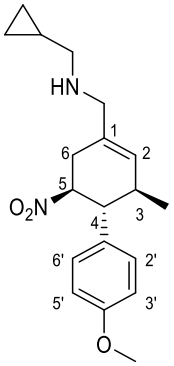
(3*S*,4*S*,5*R*)-**59** (**59a**). Following general procedure B, compound **59a** was obtained from **58a** (405 mg, 1.06 mmol) and (cyclopropylmethyl)amine (0,18 mL, 2.12 mmol) as a yellow oil (360 mg, 74%). Chromatography: hexane to hexane/EtOAc 7:3.



R_f: 0.48 (hexane/EtOAc 7:3). $[\alpha]^{D}_{20}$ = +5.3 (c = 0.51, $CHCl_3$). IR (ATR): ν 3311 (NH), 1550 (NO_2), 1251 (COC). ^{1}H-NMR ($CDCl_3$, 700 MHz): δ 0.12-0.14 (m, 2H, CH_{2cpr}), 0.49-0.52 (m, 2H, CH_{2cpr}), 0.90 (d, *J* = 7.0, 3H, CH_3), 0.94-1.00 (m, 1H, CH_{cpr}), 1.73 (br s, 1H, NH), 2.44-2.48 (m, 3H, H_3, NHC$\underline{H}_2$CH), 2.72-2.84 (m, 2H, $2H_6$), 2.82 (t, *J* = 11.1, 1H, H_4), 3.24 (AB system, *J* = 13.8, 2H, NHC$\underline{H}_2$), 3.78 (s, 3H, OCH_3), 4.96 (td, *J* = 11.1, 5.5, 1H, H_5), 5.56 (s, 1H, H_2), 6.83 (d, *J* = 8.2, 2H, $H_{3'}$, $H_{5'}$), 7.10 (d, *J* = 8.3, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.6 ($2CH_{2cpr}$), 11.3 (CH_{cpr}), 19.7 (CH_3), 33.4 (C_6), 37.5 (C_3), 51.8 (C_4), 54.6 (NH$\underline{C}H_2$CH), 54.8 ($NHCH_2$),

55.3 (OCH_3), 88.8 (C_5), 114.2 ($C_{3'}$, $C_{5'}$), 128.4 (C_2), 129.1 ($C_{2'}$, $C_{6'}$), 130.6 ($C_{1'}$), 131.8 (C_1), 159.0 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 0.90 ppm (d, CH_3) yielded NOE on 2.82 (t, H_4); irradiation of the signal at δ 2.44-2.48 ppm (m, H_3) yielded NOE on 7.10 (d, $H_{2'}$, $H_{6'}$); and irradiation of the signal at δ 4.96 ppm (td, H_5) yielded NOE on 7.10 (d, $H_{2'}$, $H_{6'}$). HPLC (t_R, min): 15.25. MS (ESI, *m/z*, %): 331.1 ($[M+H]^+$, 100).

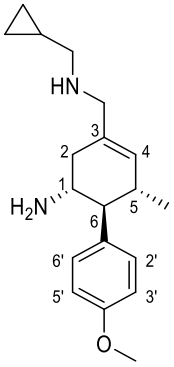
(3*R*,4*R*,5*S*)-**59** (**59b**). Following general procedure B, compound **59b** was obtained from **58b** (450 mg, 1.64 mmol) and (cyclopropylmethyl)amine (0,28 mL, 3.28 mmol) as a yellow oil (257 mg, 57%). Chromatography: hexane to hexane/EtOAc 7:3.



$[\alpha]^D_{20}$= -4.9 (c = 0.62, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **59a**.

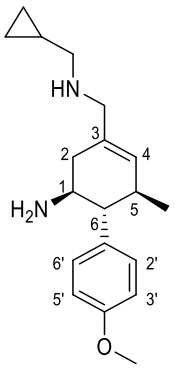
3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-methoxyphenyl)-5-methylcyclohex-3-en-1-amine, 11

(1*R*,5*S*,6*S*)-**11** (**11a**). Following general procedure C, compound **11a** was obtained from **59a** (228 mg, 0.69 mmol) as a yellow oil (190 mg, 94%). Chromatography: DCM to DCM/methanol/NH_3 9:1:0.05.



R_f: 0.04 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^D_{20}$ = +15.5 (c = 1.00, $CHCl_3$). IR (ATR): ν 3560 (NH_2), 1513(C-N), 1250 (COC). ^{1}H-NMR ($CDCl_3$, 700 MHz): δ 0.11-0.13 (m, 2H, CH_{2cpr}), 0.48-0.50 (m, 2H, CH_{2cpr}), 0.81 (d, *J* = 7.0, 3H, CH_3), 0.96-1.01 (m, 1H, CH_{cpr}), 1.93-1.97 (m, 1H, H_6), 2.00 (t, *J* = 10.4, 1H, H_2), 2.35-2.38 (m, 2H, H_2, H_5), 2.46 (dd, *J* = 7.3, 2.5, 2H, NHC$\underline{H}_2$CH), 3.16 (td, *J* = 10.5, 5.2, 1H, H_1), 3.21 (AB system, *J* = 13.8, 2H, NHC$\underline{H}_2$), 3.80 (s, 3H, OCH_3), 5.47 (s, 1H, H_4), 6.87 (d, *J* = 8.3, 2H, $H_{3'}$, $H_{5'}$), 7.11 (d, *J* = 8.3, 2H, $H_{2'}$, $H_{6'}$).^{13}C-NMR ($CDCl_3$): δ 3.5 (CH_{2cpr}), 3.6 (CH_{2cpr}), 11.4 (CH_{cpr}), 20.2 (CH_3), 37.05 (C_2), 38.1 (C_5), 51.9 (C_1), 54.5 (NH$\underline{C}H_2$CH), 55.3 ($NHCH_2$), 55.4 (OCH_3), 57.4 (C_6), 114.1 ($C_{3'}$, $C_{5'}$), 128.5 (C_4), 129.4 ($C_{2'}$, $C_{6'}$), 133.7 ($C_{1'}$), 134.7 (C_3), 158.4 ($C_{4'}$). 1D ^{1}H-NMR NOE: irradiation of the signal at δ 3.16 ppm (td, H_1) yielded NOE on 7.11 (d, $H_{2'}$, H_6'). HPLC (t_R, min): 4.00 MS (ESI, *m/z*, %): 301.1 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{19}H_{28}N_2O \cdot 2HCl \cdot 2H_2O$: %C 55.74, %H 8.37, %N 6.84; experimental: %C 56.47, %H 8.16, %N: 6.56.

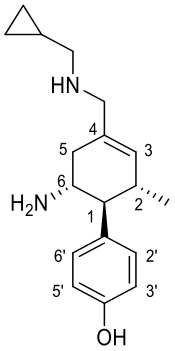
(1*S*,5*R*,6*R)*-**11** (**11b**). Following general procedure C, compound **11b** was obtained from **59b** (216 mg, 0.65 mmol) as a yellow oil (176 mg, 90%). Chromatography: DCM to DCM/methanol/NH_3 9:1:0.05.



$[\alpha]^D_{20}$= -12.2 (c = 0.99, $CHCl_3$). Elemental analysis calculated for $C_{19}H_{28}N_2O \cdot 2HCl \cdot 2H_2O$: %C 55.74, %H 8.37, %N 6.84; experimental: %C 56.36, %H 7.99, %N 6.49. Spectroscopic data were in agreement with those described for enantiomer **11a**.

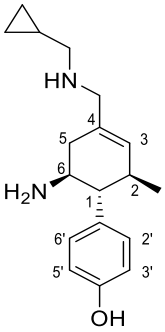
4-[6-Amino-4-{[(cyclopropylmethyl)amino]methyl}-2-methylcyclohex-3-en-1-yl]phenol, 12

(1*S*,2*S,*6*R*)-**12** (**12a**). Following general procedure F, compound **12a** was obtained from **11a** (68 mg, 0.23 mmol) as a yellow oil (43 mg, 65%).



R_f: 0.22 (DCM/methanol/NH_3 9:1:0.1). $[\alpha]^D_{20}$ = +15.9 (c = 1.26, methanol). ^{1}H-NMR (methanol-*d4*): δ 0.16-0.21 (m, 2H, CH_{2cpr}), 0.50-0.56 (m, 2H, CH_{2cpr}), 0.81 (d, *J* = 7.0, 3H, CH_3), 0.91-1.03 (m, 1H, CH_{cpr}), 1.98-2.05 (m, 2H, H_1, H_5), 2.33-2.41 (m, 2H, H_2, H_5), 2.45 (d, *J* = 6.9, 2H, NHC$\underline{H}_2$CH), 3.15 (td, *J* = 10.6, 5.2, 1H, H_6), 3.22 (s, 2H, NHC$\underline{H}_2$), 5.53 (s, 1H, H_3), 6.79 (d, *J* = 8.5, 2H, $H_{3'}$, $H_{5'}$), 7.06 (d, *J* = 8.5, 2H, $H_{2'}$, $H_{6'}$).^{13}C-NMR (methanol-*d4*): δ 4.05, 4.08 ($2CH_{2cpr}$), 11.2 (CH_{cpr}), 20.3 (CH_3), 36.7 (C_5), 39.2 (C_2), 52.7 (C_6), 54.6 (NH$\underline{C}H_2$CH), 55.4 ($NHCH_2$), 57.4 (C_1), 116.6 ($C_{3'}$, $C_{5'}$), 130.4 ($C_{2'}$, $C_{6'}$), 131.0 (C_3) 133.3, 133.6 ($C_{1'}$, C_4), 157.5 ($C_{4'}$). HPLC (t_R, min): 13.02. MS (ESI, *m/z*, %): 287.3 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{18}H_{26}N_2O \cdot 2HCl \cdot H_2O$: %C 57.29, %H 8.01, %N 7.42; experimental: %C 58.07, %H 8.46, %N 7.21.

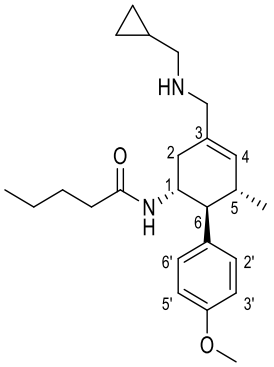
(1*R*,2*R,*6*S*)-**12** (**12b**). Following general procedure F, compound **12b** was obtained from **11b** (50 mg, 0.17 mmol) as a yellow oil (28 mg, 58%).



$[\alpha]^D_{20}$= -14.9 (c = 0.62, methanol). Elemental analysis calculated for $C_{18}H_{26}N_2O \cdot 2HCl \cdot 2H_2O$: %C 54.68, %H 8.16, %N 7.09; experimental: %C 55.28, %H 8.23, %N 6.54. Spectroscopic data were in agreement with those described for enantiomer **12a**.

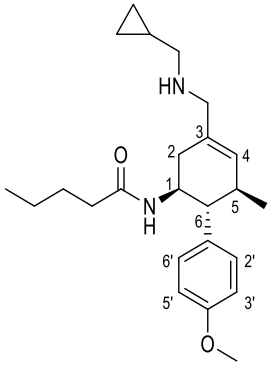
***N*-[3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-methoxyphenyl)-5-methylcyclohex-3-en-1-yl]pentanamide, 13**

(1*R*,5*S*,6*S*)-**13** (**13a**). Following general procedure G, compound **13a** was obtained from **11a** (105 mg, 0.35 mmol) and valeric acid (38 µL, 0.35 mmol) as a yellow oil (28 mg, 21%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3) 95:5:0.05.



R_f: 0.34 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^{D}_{20}$ = +20.3 (c = 0.64, $CHCl_3$). Chiral HPLC (method F, t_R, min): 8.18 .IR (ATR): ν 3282 (NH), 1640 (C=O), 1513 (C-N), 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.10-0.15 (m, 2H, CH_{2cpr}), 0.46-0.52 (m, 2H, CH_{2cpr}), 0.73 (t, *J* = 7.2, 3H, $C\underline{H}_3CH_2$), 0.83 (d, *J* = 6.9, 3H, CH_3), 0.89-1.04 (m, 3H, CH_{cpr}, $CH_3C\underline{H}_2$), 1.19-1.30 (m, 2H, $CH_2C\underline{H}_2CH_2$), 1.76-1.98 (m, 2H, $NHCOC\underline{H}_2$), 1.93-2.03 (m, 1H, H_2), 2.15-2.23 (m, 1H, H_6), 2.37-2.47 (m, 1H, H_5), 2.45 (d, *J* = 6.8, 2H, $NHC\underline{H}_2CH$), 2.59 (dd, *J* = 16.9, 5.4, 1H, H_2), 3.19 (s, 2H, $NHC\underline{H}_2$), 3.78 (s, 3H, OCH_3), 4.27-4.39 (m, 1H, H_1), 4.99 (d, *J* = 8.2, 1H, NHCO), 5.47 (s, 1H, H_4), 6.83 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.07 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$). ^{13}C-NMR ($CDCl_3$): δ 3.5 (CH_{2cpr}), 3.6 (CH_{2cpr}), 11.4 (CH_{cpr}), 13.9 ($\underline{C}H_3CH_2$), 20.2 (CH_3), 22.1 ($CH_3\underline{C}H_2$), 27.9 ($CH_2\underline{C}H_2CH_2$), 35.4 (C_2), 36.8 ($NHCO\underline{C}H_2$), 38.9 (C_5), 49.8 (C_1), 53.9 (C_6), 54.6 ($NH\underline{C}H_2CH$), 55.2 ($NHCH_2$), 55.4 (OCH_3), 113.9 ($C_{3'}$, $C_{5'}$), 128.2 (C_4), 129.3 ($C_{2'}$, $C_{6'}$), 133.6 ($C_{1'}$), 133.7 (C_3), 158.5 ($C_{4'}$), 172.6 (NHCO). HPLC (t_R, min): 15.54. MS (ESI, *m/z*, %): 385.2 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{24}H_{36}N_2O_2 \cdot HCl \cdot H_2O$: %C 65.66, %H 8.95, %N 6.38; experimental: %C 65.02, %H 8.59, %N 6.25.

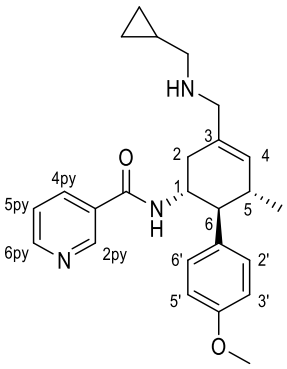
(1*S*,5*R*,6*R*)-**13** (**13b**). Following general procedure G, compound **13b** was obtained from **11b** (225 mg, 0.75 mmol) and valeric acid (81 µL, 0.75 mmol) as a yellow oil (42 mg, 18%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.05.



$[\alpha]^{D}_{20}$= -26.5 (c = 0.77, $CHCl_3$). Chiral HPLC (method F, t_R, min): 9.45. Elemental analysis calculated for $C_{24}H_{36}N_2O_2 \cdot HCl \cdot H_2O$: %C 65.66, %H 8.95, %N 6.38; experimental: %C 65.92, %H 8.54, %N 6.33. Spectroscopic data were in agreement with those described for enantiomer **13a**.

N-[3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-methoxyphenyl)-5-methylcyclohex-3-en-1-yl]pyridine-3-carboxamide, 14

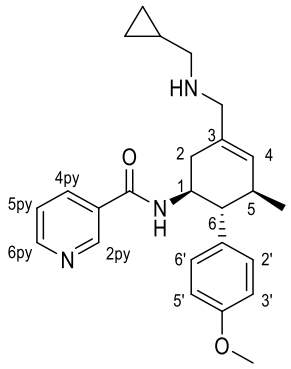
(1*R*,5*S*,6*S*)-**14** (**14a**). Following general procedure G, compound **14a** was obtained from **11a** (109 mg, 0.36 mmol) and nicotinic acid (45 mg, 0.36 mmol) as a yellow oil (37 mg, 25%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.05.



R_f: 0.29 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^{D}_{20}$ = -13.9 (c = 0.91, $CHCl_3$). Chiral HPLC (method F, t_R, min): 12.3. IR (ATR): ν 3277 (NH), 1637 (C=O), 1513 (C-N), 1248 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.11-0.16 (m, 2H, CH_{2cpr}), 0.47-0.53 (m, 2H, CH_{2cpr}), 0.88 (d, *J* = 6.9, 3H, CH_3), 0.92-1.05 (m, 1H, CH_{cpr}), 1.80 (br s, 1H, NH), 2.08-2.18 (m, 1H, H_2), 2.33-2.40 (m, 1H, H_6), 2.45-2.53 (m, 1H, H_5), 2.48 (d, *J* = 6.9, 2H, NHC$\underline{H}_2$CH), 2.77 (dd, *J* = 16.8, 5.1, 1H, H_2), 3.24 (s, 2H, NHC$\underline{H}_2$), 3.75 (s, 3H, OCH_3), 4.46 (tdd, *J* = 10.7, 7.6, 5.1, 1H, H_1), 5.53 (s, 1H, H_4), 5.79 (d, *J* = 7.6, 1H, NHCO), 6.84 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.14 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$), 7.23-7.27

(m, 1H, H_{5py}), 7.74 (dt, d, *J* = 7.9, 1.9, 1H, H_{4py}), 8.46 (d, *J* = 1.9, 1H, H_{2py}), 8.60 (dd, *J* = 4.8, 1.6, 1H, H_{6py}). ^{13}C-NMR ($CDCl_3$): δ 3.58 (CH_{2cpr}), 3.61 (CH_{2cpr}), 11.3 (CH_{cpr}), 20.2 (CH_3), 35.2 (C_2), 38.7 (C_5), 51.0 (C_1), 53.8 (C_6), 54.6 ($NH\underline{C}H_2CH$), 55.1 ($NHCH_2$), 55.3 (OCH_3), 114.2 ($C_{3'}$, $C_{5'}$), 123.5 (C_{5py}), 128.4 (C_4), 129.2 ($C_{2'}$, $C_{6'}$), 130.8 (C_{3py}), 133.1 ($C_{1'}$), 133.4 (C_3), 135.0 (C_{4py}), 147.5 (C_{2py}), 152.1 (C_{6py}), 158.7 ($C_{4'}$), 165.4 (NHCO). HPLC (t_R, min): 13.57. MS (ESI, *m/z*, %): 406.1 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{25}H_{31}N_3O_2 \cdot 2HCl \cdot 2H_2O$: %C 58.36, %H 7.25, %N 8.17; experimental: %C 59.30, %H 6.97, %N, 8.02.

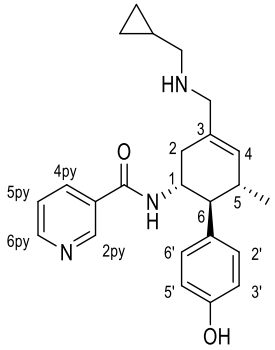
(1*S*,5*R*,6*R*)-**14** (**14b**). Following general procedure G, compound **14b** was obtained from **11b** (100 mg, 0.33 mmol) and nicotinic acid (41 mg, 0.33 mmol) as a yellow oil (49 mg, 36%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.05.



$[\alpha]^{D}_{20}$= +13.5 (c = 0.92, $CHCl_3$). HPLC (method F, t_R, min): 11.4. Elemental analysis calculated for $C_{25}H_{31}N_3O_2 \cdot 2HCl \cdot 2H_2O$: %C 58.36, %H 7.25, %N 8.17; experimental: %C 58.59, %H 7.09, %N 7.96. Spectroscopic data were in agreement with those described for enantiomer **14a**.

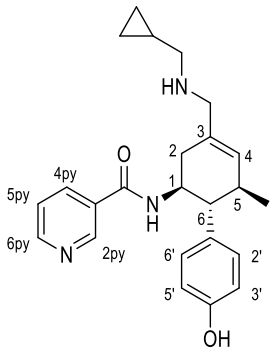
***N*-[3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-hydroxyphenyl)-5-methylcyclohex-3-en-1-yl]pyridine-3-carboxamide, 15.**

(1*R*,5*S*,6*S*)-**15** (**15a**). Following general procedure F, **15a** was obtained from **14a** (21 mg, 0.06 mmol) as a yellow oil (10 mg, 42%).



R_f: 0.42 (DCM/methanol/NH_3 9:1:0.1). $[\alpha]^D_{20}$ = +9.1 (c = 0.91, methanol). ^{1}H-NMR (methanol-*d4*): δ 0.17-0.22 (m, 2H, CH_{2cpr}), 0.50-0.56 (m, 2H, CH_{2cpr}), 0.89 (d, *J* = 6.7, 3H, CH_3), 0.94-1.04 (m, 1H, CH_{cpr}), 2.18-2.28 (m, 1H, H_2), 2.39-2.57 (m, 5H, H_2, H_5, H_6, NHC<u>H_2</u>CH), 3.25 (s, 2H, NHC<u>H_2</u>), 4.47 (td, *J* = 10.9, 5.4, 1H, H_1), 5.60 (s, 1H, H_4), 6.70 (d, *J* = 8.5, 1H, $H_{3'}$, $H_{5'}$), 7.08 (d, *J* = 8.5, 1H, $H_{2'}$, $H_{6'}$), 7.41 (app dd, *J* = 7.8, 4.9, 1H, H_{5py}), 7.81 (dt, *J* = 7.9, 1.7, 1H, H_{4py}), 8.46 (s, 1H, H_{2py}), 8.56 (app s, 1H, H_{6py}). ^{13}C-NMR (methanol-*d4*): δ 4.05 (CH_{2cpr}), 4.10 (CH_{2cpr}), 11.1 (CH_{cpr}), 20.4 (CH_3), 35.2 (C_2), 39.7 (C_5), 51.8 (C_1), 54.6 (C_6, NH<u>C</u>H_2CH), 55.3 ($NHCH_2$), 116.1 ($C_{3'}$, $C_{5'}$), 124.9 (C_{5py}), 130.4 ($C_{2'}$, $C_{6'}$), 131.2 (C_4), 132.7 (C_3), 133.5, 133.6 ($C_{1'}$, C_{3py}), 136.7 (C_{4py}), 148.6 (C_{2py}), 152.2 (C_{6py}), 157.1 ($C_{4'}$), 167.3 (NHCO). HPLC (t_R, min): 16.1. MS (ESI, *m/z*, %): 392.1 ([M + H]$^+$, 100). Elemental analysis calculated for $C_{24}H_{29}N_3O_2 \cdot 2HCl \cdot 2H_2O$: %C 57.60, %H 7.05, %N 8.40; experimental: %C 59.62, %H 7.06, %N 8.46.

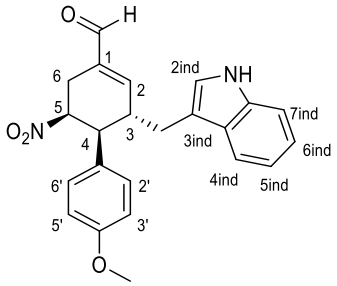
(1*S*,5*R*,6*R*)-**15** (**15b**). Following general procedure F, compound **15b** was obtained from **14b** (100 mg, 0.25 mmol) as a yellow oil (39 mg, 40%).



$[\alpha]^D_{20}$= -9.6 (c = 1.3, methanol). Elemental analysis calculated for $C_{24}H_{29}N_3O_2 \cdot 2HCl \cdot 2H_2O$: %C 57.60, %H 7.05, %N 8.40; experimental: %C 56.83, %H 6.87, %N 7.98. Spectroscopic data were in agreement with those described for enantiomer **15a**.

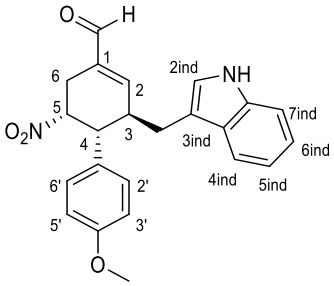
3-(1*H*-Indol-3-ylmethyl)-4-(4-methoxyphenyl)-5-nitrocyclohex-1-en-1-carbaldehyde, 60. To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.10 eq), *trans*-4-methoxy-*β*-nitrostyrene (1.00 eq) and indole (1.50 eq) in anhydrous chloroform (1 mL/mmol), a 1 M solution of acrylaldehyde in chloroform (3.00 eq) was added via a syringe pump within 12 h. The reaction mixture was stirred at rt for 24 h. Then, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (hexane to hexane/EtOAc 7:3) to afford title compound (3*S*,4*S*,5*S*)- or (3*R*,4*R*,5*R*)-**60**.

(3*S*,4*S,5S*)-**60** (**60a**). Following the previous procedure, compound **60a** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (418 mg, 2.33 mmol), using the *S* enantiomer of the catalyst (76 mg, 0.10 mmol), as a yellow solid (460 mg, 50%).



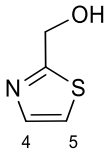
M.p.: 132-133 ºC. R_f: 0.29 (hexane/EtOAc 6:4). $[\alpha]^D_{20}$ = +68.2 (c = 0.90, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 2.78 (d, *J* = 5.8, 2H, $2H_6$), 2.86 (dd, *J* = 14.5, 8.6, 1H, ½CH_2), 3.12 (dd, *J* = 14.5, 5.2, 1H, ½CH_2), 3.38-3.41 (m, 1H, H_4), 3.41-3.51 (m, 1H, H_3), 3.80 (s, 3H, CH_3), 4.87-4.92 (m, 1H, H_5), 6.87 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 7.02-7.07 (m, 2H, H_2, H_{2ind}), 7.05 (d, *J* = 8.8, 2H, $H_{2'}$, $H_{6'}$), 7.08-7.14 (m, H_{5ind}), 7.19-7.24 (m, 1H, H_{6ind}), 7.39 (d, *J* = 8.1, 1H, H_{7ind}), 7.45 (d, *J* = 8.1, 1H, H_{4ind}), 8.11 (br s, 1H, NH), 9.49 (s, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 24.4 (C_6), 29.7 (CH_2), 40.8 (C_3), 46.9 (C_4), 55.4 (CH_3), 84.0 (C_5), 111.5 (C_{7ind}), 112.2 (C_{Ar}), 114.6 ($C_{3'}$, $C_{5'}$), 118.8 (C_{4ind}), 119.8 (C_{5ind}), 122.6, 122.8 (C_{2ind}, C_{6ind}), 127.4 (C_{Ar}), 129.0 ($C_{2'}$, $C_{6'}$), 129.5 ($C_{1'}$), 136.4 (C_{Ar}), 136.5 (C_{Ar}), 153.0 (C_2), 159.6 ($C_{4'}$), 192.6 (CHO).

(3*R*,4*R,*5*R*)-**60** (**60b**). Following the previous procedure, compound **60b** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (330 mg, 1.84 mmol), using the *R* enantiomer of the catalyst (60 mg, 0.18 mmol), as a yellow solid (355 mg, 49%).



$[\alpha]^{D}_{20}$= -57.8 (c = 1.00, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **60a**.

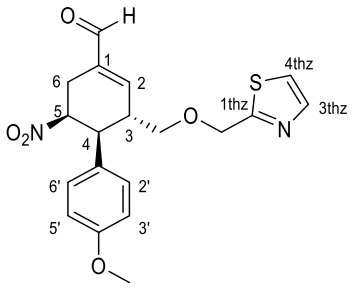
1,3-Thiazol-2-ylmethanol, 61. To a solution of 1,3-thiazol-2-carbaldehyde (0.20 mL, 2.30 mmol) in anhydrous methanol (25 mL) at 0 ºC, $NaBH_4$ (172 mg, 4.60 mmol) was added portionwise. The reaction mixture was warmed to rt and stirred for 1 h. Then, water (1 mL) was added and the solvent was evaporated under reduced pressure. The residue was suspended in water and extracted with EtOAc (x3). The organic layers were washed with brine (x2), dried over Na_2SO_4 and filtered, and concentrated under reduced pressure to yield compound **61** as a white solid (245 mg, 94%), which was used in the next step without further purification. The spectroscopic data were consistent with those previously reported.[84]



M.p.: 63 ºC (lit.[84] 63 ºC). R_f: 0.25 (hexane/EtOAc 6:4). ^{1}H-NMR ($CDCl_3$): δ 2.98 (s, 1H, OH), 4.96 (s, 2H, CH_2), 7.32 (d, *J* = 3.2, 1H, H_4), 7.74 (d, *J* = 3.2, 1H, H_5).

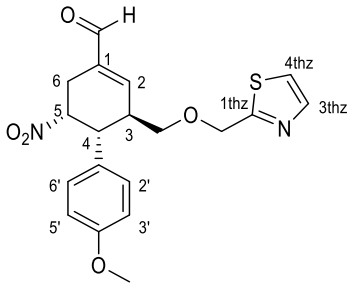
4-(4-Methoxyphenyl)-5-nitro-3-[(1,3-thiazol-2-ylmethoxy)methyl]-cyclohex-1-en-1-carbaldehyde, 62. To a solution of (*S*)- or (*R*)-2-{diphenyl[(trimethylsilyl)oxy]methyl}pyrrolidine (0.10 eq), *trans*-4-methoxy-*β*-nitrostyrene (1.00 eq) and **61** (1.10 eq) in anhydrous chloroform (1 mL/mmol), a 1 M solution of acrylaldehyde in chloroform (3.00 eq) was added via a syringe pump within 12 h. The reaction mixture was stirred at rt for 40 h. Then, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (DCM to DCM/ethanol 95:5) to yield title compound (3*S*,4*S*,5*S*)- or (3*R*,4*R*,5*R*)-**62**.

(3*S*,4*S,5S*)-**62** (**62a**). Following the previous procedure, compound **62a** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (601 mg, 3.40 mmol), using the *S* enantiomer of the catalyst (109 mg, 0.34 mmol), as a yellow solid (659 mg, 51%).



R_f: 0.40 (DCM/ethanol 95:5). $[\alpha]^D_{20}$ = +46.1 (c = 0.65, $CHCl_3$). IR (ATR): ν 1683 (C=O), 1548 (NO_2), 1253 (COC). ^{1}H-NMR ($CDCl_3$): δ 2.80-2.84 (m, 2H, $2H_6$), 3.28-3.35 (m, 1H, H_3), 3.52 (dd, *J* = 7.0, 4.1, 1H, H_4), 3.63 (dd, *J* = 9.2, 5.0, 1H, ½OCH_2), 3.76-3.81 (m, 1H, ½OCH_2), 3.77 (s, 3H, CH_3), 4.77 (AB system, *J* = 13.6, 2H, CH_2C_{thz}), 4.98-5.03 (m, 1H, H_5), 6.83 (d, *J* = 8.8, 2H, $H_{3'}$, $H_{5'}$), 6.98 (d, *J* = 8.8, 2H, $H_{2'}$, $H_{6'}$), 7.02-7.03 (m, 1H, H_2), 7.35 (d, *J* = 3.3, 1H, H_{4thz}), 7.73 (d, *J* = 3.3, 1H, H_{3thz}), 9.60 (s, 1H, CHO). ^{13}C-NMR ($CDCl_3$): δ 24.2 (C_6), 40.8 (C_3), 43.8 (C_4), 55.3 (CH_3), 70.1 ($\underline{C}H_2C_{thz}$), 71.9 (OCH_2), 84.2 (C_5), 114.5 ($C_{3'}$, $C_{5'}$), 120.0 (C_{4thz}), 128.8 ($C_{2'}$, $C_{6'}$), 129.0 ($C_{1'}$), 137.8 (C_1), 142.6 (C_{3thz}), 150.2 (C_2), 159.4 ($C_{4'}$), 167.8 (C_{1thz}), 192.4 (CHO). 1D ^{1}H-NMR NOE: irradiation of the signal at 4.98-5.03 ppm (m, H_5) yielded NOE on 2.80-2.84 (m, $2H_6$), and 3.52 (dd, H_4). HPLC (t_R, min): 22.85. MS (ESI, *m/z*, %): 389.1 ($[M+H]^+$, 100).

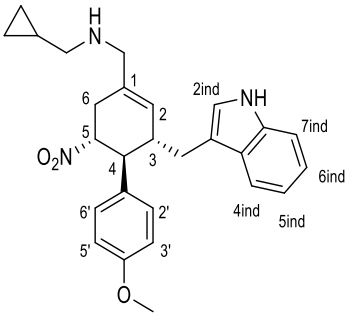
(3*R*,4*R,5R*)-**62** (**62b**). Following the previous procedure, compound **62b** was obtained from *trans*-4-methoxy-*β*-nitrostyrene (418 mg, 2.30 mmol), using the *R* enantiomer of the catalyst (76 mg, 0.23 mmol), as a yellow solid (487 mg, 54%).



$[\alpha]^D_{20}$= -61.21 (c = 0.92, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **62a**.

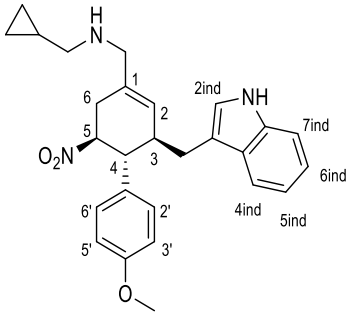
1-Cyclopropyl-*N*-{3-(1*H*-indol-3-ylmethyl)-4-(4-methoxyphenyl)-5-nitrocyclohex-1-en-1-yl]methyl}methanamine, 63

(3*S*,4*S*,5*R*)-**63** (**63a**). Following general procedure B, compound **63a** was obtained from **60a** (221 mg, 0.57 mmol) and (cyclopropylmethyl)amine (0,10 mL, 1.14 mmol) as a yellow oil (220 mg, 87%), which was used in the next step without further purification.



R_f: 0.33 (DCM/methanol 9:1). $[\alpha]^{D}_{20}$ = +16.5 (c = 0.80, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 0.04-0.09 (m, 2H, CH_{2cpr}), 0.43-0.49 (m, 2H, CH_{2cpr}), 0.83-0.96 (m, 1H, CH_{cpr}), 2.35 (d, *J* = 6.8, 2H, NHC$\underline{H}_2$CH), 2.42 (dd, *J* = 13.9, 9.9, 1H, ½CH_2C_{ind}), 2.71-2.84 (m, 3H, H_3, 2H_6), 2.90 (dd, *J* = 14.1, 2.6, 1H, ½CH_2C_{ind}), 3.07 (dd, *J* = 11.3, 10.3, 1H, H_4), 3.18 (s, 2H, NHC$\underline{H}_2$), 3.82 (s, 3H, CH_3), 4.96 (ddd, *J* = 11.4, 9.8, 6.3, 1H, H_5), 5.67 (s, 1H, H_2), 6.92 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 6.91-6.95 (m, 1H, H_{2ind}), 7.01-7.06 (m, 1H, H_{6ind}), 7.13-7.24 (m, 2H, H_{5ind}, H_{7ind}), 7.24 (d, *J* = 8.7, 2H, $H_{2'}$, $H_{6'}$), 7.33 (d, *J* = 8.1, 2H, H_{4ind}), 8.01 (NH_{ind}). ^{13}C-NMR ($CDCl_3$): δ 3.5 (2CH_{2cpr}), 11.3 (CH_{cpr}), 28.8 ($\underline{C}H_2C_{ind}$), 33.2 (C_6), 43.1 (C_3), 50.1 (C_4), 54.5 (NH$\underline{C}H_2$CH), 55.0 ($NHCH_2$), 55.4 (CH_3), 89.11 (C_5), 111.2 (C_{4ind}), 113.5 (C_{3ind}), 114.4 ($C_{3'}$, $C_{5'}$), 119.0 (C_{7ind}), 119.4 (C_{6ind}), 122.14 (C_{5ind}), 122.5 (C_{2ind}), 126.2 (C_2), 127.6 ($C_{7a\ ind}$), 129.4 ($C_{2'}$, $C_{6'}$), 130.7 ($C_{1'}$), 132.2 (C_1), 136.4 ($C_{3a\ ind}$), 159.2 ($C_{4'}$). HPLC (t_R, min): 19.50. MS (ESI, *m/z*, %): 446.3 ($[M+H]^+$, 100).

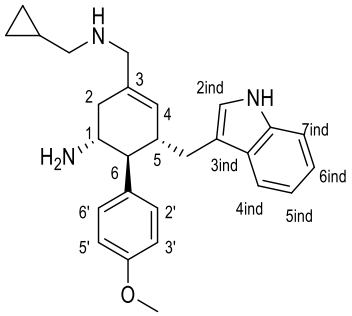
(3*R*,4*R*,5*S*)-**63** (**63b**). Following general procedure B, compound **63b** was obtained from **60b** (450 mg, 1.15 mmol) and (cyclopropylmethyl)amine (0,20 mL, 2.30 mmol) as a yellow oil (420 mg, 82%), which was used in the next step without further purification.



$[\alpha]^{D}_{20}$= -21.4 (c = 0.54, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **63a**.

3-{[(Cyclopropylmethyl)amino]methyl}-5-(1*H*-indol-3-ylmethyl)-6-(4-methoxyphenyl)cyclohex-3-en-1-amine, 16

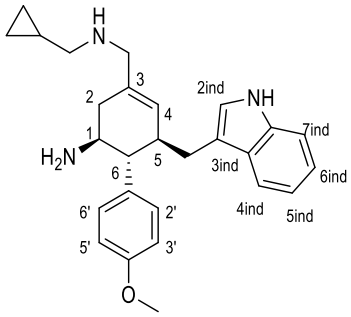
(1*R*,5*S*,6*S*)-**16** (**16a**). Following general procedure C, compound **16a** was obtained from **63a** (170 mg, 0.38 mmol) as a white solid (137 mg, 87%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 9:1:0.1.



M.p.: 144-145 ºC. R_f: 0.20 (DCM/methanol/NH_3 9:1:0.1). $[\alpha]^{D}_{20}$ = +18.9 (c = 0.75, $CHCl_3$). Chiral HPLC (method I, t_R, min): 7.29. ^{1}H-NMR ($CDCl_3$): δ 0.04-0.09 (m, 2H, CH_{2cpr}), 0.42-0.48 (m, 2H, CH_{2cpr}), 0.83-0.97 (m, 1H, CH_{cpr}), 1.92-2.01 (m, 1H, H_2), 2.25 (t, *J* = 10.5, 1H, H_6), 2.28-2.41 (m, 2H, ½CH_2C_{ind}, H_2), 2.37 (d, *J* = 7.0, 2H, NHC$\underline{H}_2$CH), 2.66-2.74 (m, 1H, H_5), 2.81 (dd, *J* = 14.2, 2.6, 1H, ½CH_2C_{ind}), 3.16 (s, 2H, NHC$\underline{H}_2$), 3.16-3.23 (m, 1H, H_1), 3.84 (s, 3H, CH_3), 5.59 (s, 1H, H_4), 6.92-6.94 (m, 1H, H_{2ind}), 6.95 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 6.98-7.04 (m, 1H, H_{6ind}), 7.10-7.16 (m, 1H, H_{5ind}), 7.21-7.26 (m, 1H, H_{7ind}), 7.25 (d, *J* = 8.8, 2H, $H_{2'}$, $H_{6'}$), 7.31 (d, *J* = 8.1, 1H, H_{4ind}), 8.08 (s, 1H, NH_{ind}). ^{13}C-NMR ($CDCl_3$): δ 3.6 ($2CH_{2cpr}$), 11.1 (CH_{cpr}), 29.2 ($\underline{C}H_2C_{ind}$), 36.9 (C_2), 43.7 (C_5), 52.2 (C_1), 54.2 (NH$\underline{C}H_2$CH), 55.3 ($NHCH_2$), 55.40 (CH_3), 55.48 (C_6), 112.1 (C_{4ind}), 114.36 ($C_{3'}$, $C_{5'}$), 114.42 (C_{3ind}), 119.07, 119.14 (C_{6ind}, C_{7ind}), 121.8 (C_{4ind}), 122.3 (C_{2ind}), 126.6 (C_4), 127.9 ($C_{7a\ ind}$), 129.8 ($C_{2'}$, $C_{6'}$), 133.6 (C_3), 134.6 ($C_{1'}$), 136.4 ($C_{3a\ ind}$), 158.63 ($C_{4'}$). HPLC (t_R, min):

13.34. MS (ESI, *m/z*, %): 416.3 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{27}H_{33}N_3O \cdot 2HCl \cdot 2H_2O$: %C 61.83, %H 7.49, %N 8.01; experimental: %C 60.58, %H 7.08, %N 7.61.

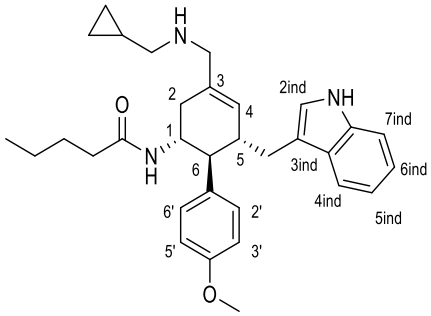
(1*S*,5*R*,6*R)*-**16** (**16b**). Following general procedure C, compound **16b** was obtained from **63b** (190 mg, 0.43 mmol) as a white solid (172 mg, 95%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 9:1:0.1.



$[\alpha]^D_{20}$= -20.8 (c = 1.06, $CHCl_3$). Chiral HPLC (method I, t_R, min): 6.14. Elemental analysis calculated for $C_{27}H_{33}N_3O \cdot 2HCl \cdot 2H_2O$: %C 61.83, %H 7.49, %N 8.01; experimental: %C 62.23, %H 7.40, %N 7.68. Spectroscopic data were in agreement with those described for enantiomer **16a**.

N-[3-{[(Cyclopropylmethyl)amino]methyl}-5-(1*H*-indol-3-ylmethyl)-6-(4-methoxyphenyl)cyclohex-3-en-1-yl]pentanamide, 17

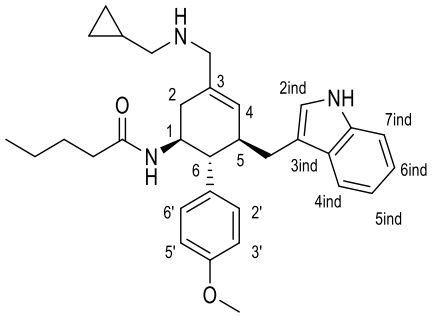
(*1R*,5*S*,6*S*)-**17** (**17a**). Following general procedure G, compound **17a** was obtained from **16a** (213 mg, 0.51 mmol) and valeric acid (60 µL, 0.51 mmol) as a yellow oil (40 mg, 16%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 92:8:0.5.



R_f: 0.40 (: DCM to DCM/methanol/NH_3 9:1:0.05). $[\alpha]^D_{20}$ = +25.6 (c = 0.75, $CHCl_3$). Chiral HPLC (method I, t_R, min): 6.97. ^{1}H-NMR ($CDCl_3$): δ 0.06-0.10 (m, 2H, CH_{2cpr}), 0.42-0.49(m, 2H, CH_{2cpr}), 0.74 (t, *J* = 7.2, 3H, $C\underline{H}_3CH_2$), 0.86-1.05 (m,

3H, CH_{cpr}, $CH_3C\underline{H}_2$.), 1.19-1.32 (m, 3H, $CH_2C\underline{H}_2CH_2$, NH), 1.76-2.01 (m, 3H, $C\underline{H}_2CO$, H_2), 2.32-2.48 (m, 2H, ½CH_2C_{ind}, H_6), 2.38 (d, *J* = 6.9, 2H, $NHC\underline{H}_2CH$), 2.57 (dd, *J* = 16.9, 4.6, 1H, H_2), 2.73-2.87 (m, H_5, ½CH_2C_{ind}), 3.15 (s, 2H, $NHC\underline{H}_2$), 3.81 (s, 1H, OCH_3), 4.31 (tdd, *J* = 10.6, 8.3, 5.3, 1H, H_1), 5.07 (d, *J* = 8.1, NHCO), 5.61 (s, 1H, H_4), 6.90 (d, *J* = 8.7, 2H, $H_{3'}$, $H_{5'}$), 6.93 (d, *J* = 2.0, 1H, H_{2ind}), 7.01 (td, *J* = 7.4, 0.8, 1H, H_{6ind}), 7.10-7.22 (m, 2H, H_{5ind}, H_{7ind}), 7.19 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$), 7.31 (d, *J* = 8.1, 1H, H_{4ind}), 8.14 (s, 1H, NH_{ind}). ^{13}C-NMR ($CDCl_3$): δ 3.6 ($2CH_{2cpr}$), 11.1 (CH_{cpr}), 13.9 ($\underline{C}H_3CH_2$), 22.1 ($CH_3\underline{C}H_2$), 27.9 ($CH_2\underline{C}H_2CH_2$), 29.2 ($C\underline{H}_2C_{ind}$), 35.3 (C_2), 36.8 ($\underline{C}H_2CO$), 44.4 (C_5), 50.2 (C_1), 52.0 (C_6), 54.3 ($NHC\underline{H}_2CH$), 55.2 ($NHCH_2$), 55.4 (OCH_3), 111.2 (C_{4ind}), 114.16 (C_{3ind}), 114.19 ($C_{3'}$, $C_{5'}$), 119.1, 119.2 (C_{6ind}, C_{7ind}), 121.9 (C_{5ind}), 122.3 (C_{2ind}), 126.4 (C_4), 127.9 ($C_{7a\ ind}$), 129.6 ($C_{2'}$, $C_{6'}$), 133.6 (C_3, $C_{1'}$), 136.4 ($C_{3a\ ind}$), 158.8 ($C_{4'}$), 172.6 (NHCO). HPLC (t_R, min): 17.63. MS (ESI, *m/z*, %): 500.4 ([M+H]$^+$, 100). Elemental analysis calculated for $C_{32}H_{41}N_3O_2 \cdot HCl \cdot 2H_2O$: %C 67.17, %H 8.10, %N 7.34; experimental: %C 66.64, %H 7.90, %N 7.34.

(*1S*,5*R*,6*R*)-**17** (**17b**). Following general procedure G, compound **17b** was obtained from **16b** (125 mg, 0.30 mmol) and valeric acid (30 µL, 0.30 mmol) as a yellow oil (19 mg, 13%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 92:8:0.5.

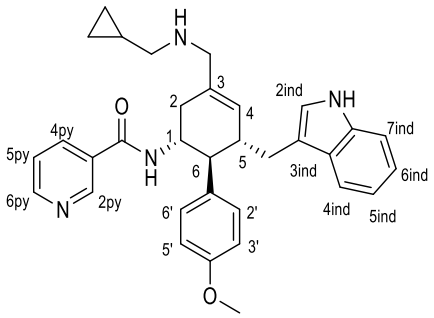


$[\alpha]^D_{20}$= -36.5 (c = 0.41, $CHCl_3$). Chiral HPLC (method I, t_R, min): 8.05. Elemental analysis calculated for $C_{32}H_{41}N_3O_2 \cdot HCl \cdot 2H_2O$: %C 67.17, %H 8.10, %N 7.34; experimental: %C 65.94, %H 7.79, %N 7.20. Spectroscopic data were in agreement with those described for enantiomer **17a**.

N-[3-{[(Cyclopropylmethyl)amino]methyl}-5-(1*H*-indol-3-ylmethyl)-6-(4-methoxyphenyl)cyclohex-3-en-1-yl]nicotinamide, 18

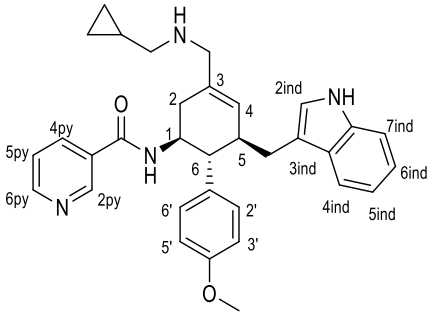
(1*R*,5*S*,6*S*)-**18** (**18a**). Following general procedure G, compound **18a** was obtained from **16a** (130 mg, 0.31 mmol) and nicotinic acid (38 mg, 0.31 mmol) as

a yellow oil (39 mg, 23%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 92:8:0.5.



R_f: 0.42 (DCM/methanol/NH_3 9:1:0.05). $[\alpha]^D_{20}$ = +7.7 (c = 0.70, $CHCl_3$). Chiral HPLC (method I, t_R, min): 7.78. ^{1}H-NMR ($CDCl_3$): δ 0.09-0.14 (m, 2H, CH_{2cpr}), 0.44-0.50 (m, 2H, CH_{2cpr}), 0.85-1.00 (m, 1H, CH_{cpr}), 2.14-2.23 (m, 1H, H_2), 2.38-2.46 (m, 1H, ½ CH_2C_{ind}), 2.43 (d, *J* = 6.8, 2H, NHC$\underline{H}_2$CH), 2.63 (t, *J* = 10.8, 1H, H_6), 2.72 (dd, *J* = 16.8, 5.1, 1H, H_2), 2.80-2.90 (m, 2H, H_5, ½ CH_2C_{ind}), 3.21 (s, 2H, NHC$\underline{H}_2$), 3.78 (s, 3H, CH_3), 4.39-4.51 (m, 1H, H_1), 5.69 (s, 1H, H_4), 6.04 (d, *J* = 7.8, NHCO), 6.89 (d, *J* = 8.6, $H_{3'}$, $H_{5'}$), 6.97 (d, *J* = 1.6, 1H, H_{2ind}), 7.02 (td, *J* = 7.5, 0.8 1H, H_{6ind}), 7.13 (td, *J* = 7.5, 0.9, 1H, H_{5ind}), 7.20-7.26 (m, 2H, H_{7ind}, H_{5py}), 7.24 (d, *J* = 8.3, $H_{2'}$, $H_{6'}$), 7.31 (d, *J* = 8.1, 1H, H_{4ind}), 7.73 (dt, *J* = 7.9, 1.9, 1H, H_{4py}), 8.34 (s, 1H, NH_{ind}), 8.51 (d, *J* = 1.7, 1H, H_{2py}), 8.59 (dd, *J* = 4.8, 1.6, 1H, H_{6py}). ^{13}C-NMR ($CDCl_3$): δ 3.71 (CH_{2cpr}) , 3.74 (CH_{2cpr}), 10.6 (CH_{cpr}), 29.1 ($\underline{C}H_2C_{ind}$), 34.9 (C_2), 44.1 (C_5), 51.3 (C_1), 51.6 (C_6), 54.1 (NHC$\underline{H}_2$CH), 54.9 ($NHCH_2$), 55.4 (CH_3), 111.2 (C_{4ind}), 113.8 (C_{3ind}), 114.4 ($C_{3'}$, $C_{5'}$), 119.1, 119.2 (C_{6ind}, C_{7ind}), 121.9 (C_{5ind}), 122.6 (C_{2ind}), 123.5 (C_{5py}), 127.4 (C_4), 127.8 ($C_{7a\ ind}$), 129.5 ($C_{2'}$, $C_{6'}$), 130.8 (C_{3py}), 132.7 (C_3), 133.2 ($C_{1'}$), 135.1 (C_{4py}), 136.4 ($C_{3a\ ind}$), 147.6 (C_{2py}), 152.0 (C_{6py}), 158.8 ($C_{4'}$), 165.4 (NHCO). HPLC (t_R, min): 15.9. MS (ESI, *m/z*, %): 521.3 ($[M+H]^+$, 100). Elemental analysis calculated for $C_{33}H_{36}N_4O_2 \cdot 2HCl \cdot 3H_2O$: %C 61.20, %H 6.85, %N 8.65; experimental: %C 61.74, %H 6.78, %N 8.42.

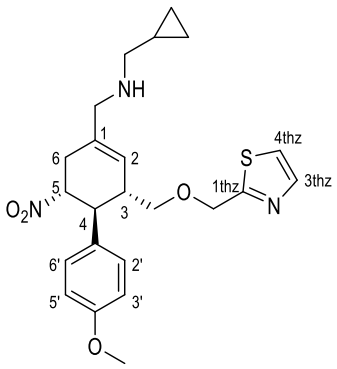
(1*S*,5*R*,6*R*)-**18** (**18b**). Following general procedure G, compound **18b** was obtained from **16b** (103 mg, 0.25 mmol) and nicotinic acid (30 mg, 0.25 mmol) as a yellow oil (39 mg, 30%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 92:8:0.5.



$[\alpha]^{D}_{20}$= -6.7 (c = 1.03, $CHCl_3$). Chiral HPLC (method I, t_R, min): 10.48. Elemental analysis calculated for $C_{33}H_{36}N_4O_2 \cdot 2HCl \cdot 3H_2O$: %C 61.20, %H 6.85, %N 8.65; experimental: %C 63.67, %H 6.94, %N 8.79. Spectroscopic data were in agreement with those described for enantiomer **18a**.

(Cyclopropylmethyl)(4-(4-methoxyphenyl)-5-nitro-3-[(1,3-thiazol-2-ylmethoxy)methyl]cyclohex-1-en-1-yl}methyl)amine, 64

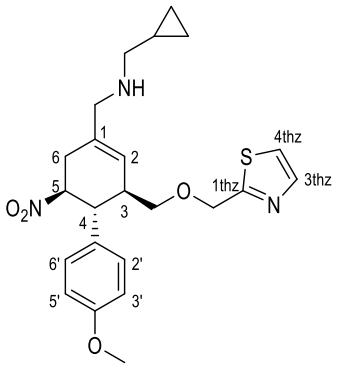
(3*S*,4*S*,5*R*)-**64** (**64a**). Following general procedure B, compound **64a** was obtained from **62a** (247 mg, 0.64 mmol) and (cyclopropylmethyl)amine (0.11 mL, 1.28 mmol) as a yellow oil (186 mg, 66%), which was used in the next step without further purification.



R_f: 0.11 (DCM/ethanol 95:5). $[\alpha]^{D}_{20}$ = +24.9 (c = 0.65, $CHCl_3$). IR (ATR): ν 1550 (NO_2), 1513 (C-N), 1251 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.21-0.26 (m, 2H, CH_{2cpr}), 0.54-0.60 (m, 2H, CH_{2cpr}), 1.03-1.11 (m, 1H, CH_{cpr}), 2.61 (d, *J* = 7.0, 2H, NHC$\underline{H_2}$CH), 2.65-2.73 (m, 1H, H_3), 2.82-2.89 (m, 2H, $2H_6$), 3.25 (t, *J* = 11.3, 1H, H_4), 3.30 (dd, *J* = 8.9, 5.8, 1H, ½OCH_2), 3.42 (s, 2H, NHC$\underline{H_2}$), 3.48 (dd, *J* = 8.9, 3.2, 1H, ½OCH_2), 3.76 (s, 3H, CH_3), 4.69 (AB system, *J* = 13.5, 2H, CH_2C_{thz}), 5.02 (ddd, *J* = 11.5, 10.1, 6.2, 1H, H_5), 5.89 (s, 1H, H_2), 6.81 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.09 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$), 7.33 (d, *J* = 3.2, 1H, H_{4thz}), 7.72 (d, *J* = 3.2, 1H, H_{3thz}).

^{13}C-NMR ($CDCl_3$): δ 4.0 ($2CH_{2cpr}$), 9.8 (CH_{cpr}), 33.1 (C_6), 43.5 (C_3), 45.5 (C_4), 53.3 ($NH\underline{C}H_2CH$), 53.5 ($NHCH_2$), 55.3 (CH_3), 70.2 ($\underline{C}H_2C_{thz}$), 71.6 (OCH_2), 88.3 (C_5), 114.4 ($C_{3'}$, $C_{5'}$), 119.7 (C_{4thz}), 127.8 (C_2), 129.1 ($C_{2'}$, $C_{6'}$), 129.6 ($C_{1'}$), 133.2 (C_1), 142.6 (C_{3thz}), 159.3 ($C_{4'}$), 168.4 (C_{1thz}). HPLC (t_R, min): 17.91. MS (ESI, *m/z*, %): 444.2 ($[M+H]^+$, 100).

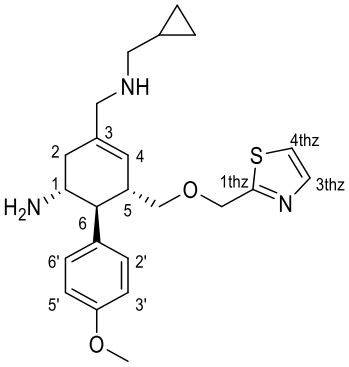
(3*R*,4*R*,5*S*)-**64** (**64b**). Following general procedure B, compound **64b** was obtained from **62b** (237 mg, 0.61 mmol) and (cyclopropylmethyl)amine (0.11 mL, 1.22 mmol) as a yellow oil (206 mg, 76%), which was used in the next step without further purification.



$[\alpha]^{D}_{20}$= -29.2 (c = 1.03, $CHCl_3$). Spectroscopic data were in agreement with those described for enantiomer **64a**.

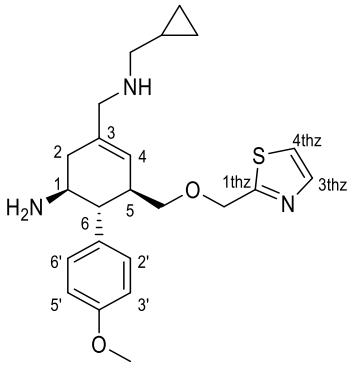
3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-methoxyphenyl)-5-[(1,3-thiazol-2-ylmethoxy)methyl]cyclohex-3-en-1-amine, 19

(1*R*,5*S*,6*S*)-**19** (**19a**). Following general procedure C, compound **19a** was obtained from **64a** (219 mg, 0.49 mmol) as a yellow oil (160 mg, 78%). Chromatography: DCM to DCM/ethanol/NH_3 9:1:0.1.



R_f: 0.12 (DCM/ethanol/NH_3 9:1:0.1). $[\alpha]^D_{20}$ = +10.2 (c = 1.61, $CHCl_3$). IR (ATR): ν 1512 (C-N), 1249 (COC)**.** ^{1}H-NMR ($CDCl_3$): δ 0.09-0.14 (m, 2H, CH_{2cpr}), 0.45-0.51 (m, 2H, CH_{2cpr}), 0.93-1.03 (m, 1H, CH_{cpr}), 1.92-2.03 (m, 1H, H_2), 2.32 (t, *J* = 10.7, 1H, H_6), 2.36-2.49 (m, 1H, H_2), 2.46 (dd, *J* = 6.9, 1.2, 2H, NHC$\underline{H}_2$CH), 2.56-2.62 (m, 1H, H_5), 3.15-3.29 (m, 2H, H_1, ½OCH_2), 3.25 (s, 2H, NHC$\underline{H}_2$), 3.42 (dd, *J* = 8.9, 3.3, 1H, ½OCH_2), 3.80 (s, 3H, CH_3), 4.68 (AB system, *J* = 13.6, 2H, CH_2C_{thz}), 5.74 (s, 1H, H_4), 6.86 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.11 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$), 7.30 (d, *J* = 3.2, 1H, H_{4thz}), 7.70 (d, *J* = 3.2, 1H, H_{3thz}). ^{13}C-NMR ($CDCl_3$): δ 3.50 (CH_{2cpr}), 3.53 (CH_{2cpr}), 11.4 (CH_{cpr}), 36.9 (C_2), 44.1 (C_5), 51.2 (C_6), 51.9 (C_1), 54.4 (NH$\underline{C}H_2$CH), 55.4 ($NHCH_2$), 55.4 (CH_3), 70.2 ($\underline{C}H_2C_{thz}$), 73.4 (OCH_2), 114.3 ($C_{3'}$, $C_{5'}$), 119.4 (C_{4thz}), 124.3 (C_4), 129.4 ($C_{2'}$, $C_{6'}$), 133.8 ($C_{1'}$), 135.8 (C_3), 142.4 (C_{3thz}), 158.6 ($C_{4'}$), 169.2 (C_{1thz}). HPLC (t_R, min): 14.85. MS (ESI, *m/z*, %): 413.7 ($[M]^+$, 100). Elemental analysis calculated for $C_{23}H_{31}N_3O_2S{\cdot}3HCl{\cdot}H_2O$: %C 51.06, %H 6.71, %N 7.77, %S 5.93; experimental: %C 52.42, %H 6.60, %N 7.80, %S 5.71.

(1*S*,5*R*,6*R*)-**19** (**19b**). Following general procedure C, compound **19b** was obtained from **64b** (128 mg, 0.29 mmol) as a yellow oil (111 mg, 93%). Chromatography: DCM to DCM/ethanol/NH_3 9:1:0.1.

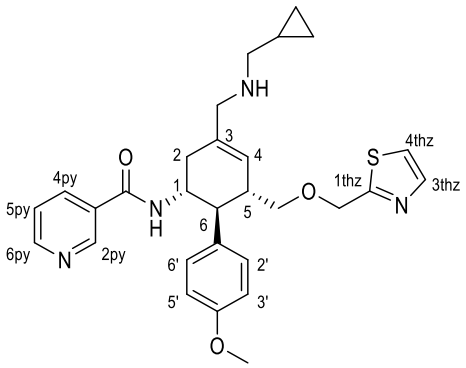


$[\alpha]^D_{20}$= -7.2 (c = 0.67, $CHCl_3$). Elemental analysis calculated for $C_{23}H_{31}N_3O_2S{\cdot}3HCl{\cdot}H_2O$: %C 51.06, %H 6.71, %N 7.77, %S 5.93; experimental: %C 52.42, %H 6.60, %N 7.80, %S 5.71. Spectroscopic data were in agreement with those described for enantiomer **19a**.

***N*-{3-{[(Cyclopropylmethyl)amino]methyl}-6-(4-methoxyphenyl)-5-[(1,3-thiazol-2-ylmethoxy)methyl]cyclohex-3-en-1-yl)nicotinamide, 20**

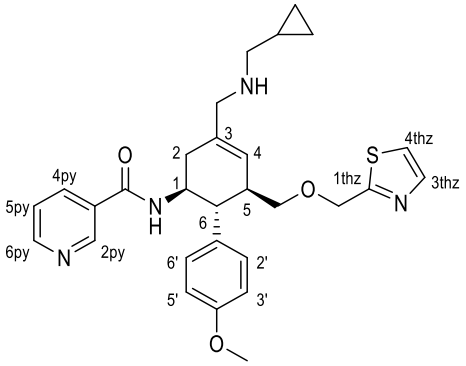
(1*R*,5*S*,6*S*)-**20** (**20a**). Following general procedure G, compound **20a** was obtained from **19a** (282 mg, 0.68 mmol) and nicotinic acid (84 mg, 0.68 mmol) as

a yellow oil (82 mg, 23%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.1.



R_f: 0.10 (DCM/methanol/NH_3 95:5:0.1). $[\alpha]^{D}_{20}$ = +13.6 (c = 1.29, $CHCl_3$). Chiral HPLC (method F, t_R, min): 9.77. IR (ATR): ν 3281 (NH), 1643 (C=O), 1512 (NH), 1253 (COC). ^{1}H-NMR ($CDCl_3$): δ 0.08-0.13 (m, 2H, CH_{2cpr}), 0.44-0.50 (m, 2H, CH_{2cpr}), 0.90-1.03 (m, 1H, CH_{cpr}), 2.12-2.21 (m, 1H, H_2), 2.47 (d, *J* = 6.6, 2H, NHC$\underline{H_2}$CH), 2.68-2.77 (m, 2H, H_2, H_5), 2.81 (t, *J* = 10.2, 1H, H_6), 3.26 (s, 2H, NHC$\underline{H_2}$), 3.33 (dd, *J* = 8.9, 5.4, 1H, ½OCH_2), 3.50 (dd, *J* = 8.9, 2.9, 1H, ½OCH_2), 3.74 (s, 3H, CH_3), 4.44-4.55 (m, 1H, H_1), 4.70 (AB system, *J* = 13.6, 2H, CH_2C_{thz}), 5.75 (s, 1H, H_4), 6.07 (d, *J* = 7.7, 1H, NHCO), 6.81 (d, *J* = 8.6, 2H, $H_{3'}$, $H_{5'}$), 7.12 (d, *J* = 8.6, 2H, $H_{2'}$, $H_{6'}$), 7.24 (app dd, *J* = 7.9, 5.4, 1H, H_{5py}), 7.31 (d, *J* = 3.3, 1H, H_{4thz}), 7.71 (d, *J* = 3.3, 1H, H_{3thz}), 7.76 (app dt, *J* = 7.9, 1.9, 1H, H_{4py}), 8.51 (d, *J* = 1.6, 1H, H_{2py}), 8.60 (dd, *J* = 4.7, 1.3, 1H, H_{6py}). ^{13}C-NMR ($CDCl_3$): δ 3.53 (CH_{2cpr}), 3.57 (CH_{2cpr}) 11.3 (CH_{cpr}), 34.4 (C_2), 44.4 (C_5), 47.4 (C_6), 50.6 (C_1), 54.4 (NH$\underline{C}H_2$CH), 55.2 (NHCH_2), 55.3 (CH_3), 70.2 ($\underline{C}H_2C_{thz}$), 72.9 (OCH_2), 114.4 ($C_{3'}$, $C_{5'}$), 119.6 (C_{4thz}), 123.5 (C_{5py}), 124.3 (C_4), 129.2 ($C_{2'}$, $C_{6'}$), 130.8 (C_{3py}), 132.6 ($C_{1'}$), 135.0 (C_{4py}), 135.7 (C_3), 142.6 (C_{3thz}), 147.6 (C_{2py}), 152.0 (C_{6py}), 158.8 ($C_{4'}$), 165.3 (CO), 168.7 (C_{1thz}). HPLC (t_R, min): 19.09. MS (ESI, *m/z*, %): 518.6 ($[M]^+$, 100). Elemental analysis calculated for $C_{29}H_{34}N_4O_3S{\cdot}3HCl{\cdot}2H_2O$: %C 52.45, %H 6.22, %N 8.44, %S 4.83; experimental: %C 53.46, %H 6.16, %N 8.30, %S 4.62.

(1*S*,5*R*,6*R*)-**20** (**20b**). Following general procedure G, **20b** was obtained from **19b** (145 mg, 0.35 mmol) and nicotinic acid (43 mg, 0.35 mmol) as a yellow oil (55 mg, 30%, 99% *ee*). Chromatography: DCM to DCM/methanol/NH_3 95:5:0.1.



$[\alpha]^{D}_{20}$= -6.3 (c = 1.00, $CHCl_3$). Chiral HPLC (method F, t_R, min): 10.86. Elemental analysis calculated for $C_{29}H_{34}N_4O_3S{\cdot}3HCl{\cdot}2H_2O$: %C 52.45, %H 6.22, %N 8.44, %S 4.83; experimental: %C 52.68, %H 6.23, %N 8.15, %S 4.60 Spectroscopic data were in agreement with those described for enantiomer **20a**.

4.2. Biological experiments

Cell lines and culture. Colon cancer cell line (HCT-116) from European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), and breast cancer cell line (MCF-7) from American Type Culture Collection (ATTC, Rockville, MD) were grown in Dulbecco's Modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 10 U/mL penicillin and 10 µg/mL streptomycin (Invitrogen). Fibroblast cell line (IMR90) was obtained from ATCC and maintained in DMEM supplemented with 15% heat-inactivated FBS, 10 U/mL penicillin, and 10 µg/mL streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere at 37 ºC with 5% CO_2.

For CSC enrichment, the cells were grown in DMEM/F12 without serum (Invitrogen), supplemented with 2% B27® (Gibco), 1% L-glutamine (Life Techonogies), 10 U/mL penicillin, 10 µg/mL streptomycin (Invitrogen), 0.5% methylcellulose (R&D Systems), 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), and basic fibroblastic growth factor (bFGF, Sigma-Aldrich).

Isolation of $CD34^+$ cells. Briefly, bone marrow samples were collected from AML and healthy patient pelvic bone, and were subjected to Ficoll-Paque density gradient separation to isolate mononuclear cells. Then, $CD34^+$ cells were

separated using high-gradient magnetic-activated cell sorting according to the manufacturer's manual, and maintained in DMEM medium supplemented with 10% FBS until their use.

4.2.1. MTT cytotoxicity assay

The sensitivity of HCT-116, MCF-7 and IMR90 cell lines to compounds was tested through a standard MTT assay. Briefly, cells were seeded in 96-well plates at a density of 5 x 10^3 (HCT-116) or 10 x 10^3 (MCF-7, IMR90) cells per well in the corresponding medium with 10 or 15% FBS for 24 h prior to treatments. The medium was then replaced by fresh medium containing tested compound or the equivalent volume of DMSO. After 48 h, the medium was replaced by fresh medium with 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich), and the cells were incubated for 4 h at 37 ºC in the dark. The supernatants were removed, formazan crystals were dissolved in DMSO (100 µL/well), and the absorbance was measured at 570 nm (OD570-630) using an Asys UVM 340 microplate reader (Biochrom Ltd., Cambridge, UK). The background absorbance of blank wells containing only medium with compound or vehicle were subtracted from each test well. The results were reported as cytotoxicity percentage of tested compound relative to vehicle, obtained from two or three independent experiments performed in triplicate.

4.2.2. Sphere-forming assay

HCT-116 and MCF-7 cell lines were seeded at a density of 2 x 10^3 cells per well on non-adherent 12-well plates (Corning Gentest) previously treated with poly(2-hydroxyethyl methacrylate) (PolyHEMA, Sigma-Aldrich), and grown in CSC medium, supplemented every 48 h with 20 ng/mL EGF and bFGF. Then, cells were treated tested compound or the equivalent volume of DMSO. After 10 days, the resulting spheres were quantified under an optical microscope and photographed using an Olympus FV1200 confocal laser microscope or a phase contrast microscope with digital camera (CAI de Citometría y Microscopía de Fluorescencia, UCM). The data were presented as percentage of inhibition of sphere formation relative to DMSO, obtained from two or three independent experiments performed in duplicate.

4.2.3. CFU assay

Sorted $CD34^+$ cells were plated in 24-well plates at a density of 3×10^4 cells per well in methylcellulose-based medium MethoCult GF H4434 (StemCell Technologies) containing stem cell factor (SCF), interleukin-3 (IL-3), erythropoietin (EPO) and granulocyte macrophage colony stimulating factor (GM-CSF). Test compound was added directly to the cells within the methylcellulose medium, and incubated at 37 °C in a humidified atmosphere in the presence of 5% CO_2 for 14 days. Total colonies formed were counted manually using an inverted microscope. The data were presented as percentage of colonies or percentage of viable cells relative to DMSO, obtained from two or three independent experiments performed in duplicate.

4.2.4. Flow cytometry

Total colonies formed in 4.2.3 were collected, and resuspended in 300 µL of annexin V binding buffer (Biolegend). The cells were incubated in the dark at rt for 30 min with antibodies against the surface markers: CD34-PE (BD Bioscience), CD71-FITC (BD BioScience) and CD45-PerCP (BD Bioscience). Annexin V-APC (Biolegend) was used to determine the cellular apoptotic population. The analysis of $CD34^+$ $CD71^+$, $CD45^+$ and annexin V^+ subpopulation was carried out in a flow cytometer FACSCalibur (Becton Dickinson, CAI Citometría y Microscopía de Fluorescencia, UCM) and the data were processed using FlowJo_V10 software. Annexin V^- cells were scored as viable cells, and annexin V^+ cells were scored as apoptotic cells.

4.2.5. WST-1 cytotoxicity assay

Sorted $CD34^+$ cells were plated in 96-well plates at a density of 5×10^4 per well. Then, test compound or the equivalent volume of DMSO was added. After 24 or 48 h of treatment, 10 µL of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt, Sigma-Aldrich) were added in each well, and the cells were incubated in the dark for 4 h at 37 ºC. Then, the absorbance was measured at 450 nm using an Asys UVM 340 microplate reader (Biochrom Ltd., Cambridge, UK). The background absorbance of blank wells containing only medium were subtracted from each test well. The data were

reported as cytotoxicity percentage relative to DMSO, obtained from two or three independent experiments performed in triplicate.

4.2.6. Pharmacokinetic study in vivo.

For pharmacokinetic studies, tested compound was administered (25 mg/Kg, ip) and blood was collected at the selected time points post-dose (n = 3 per time point) by cardiac puncture. Blood was allowed to clot at rt for 30 min and centrifuged at 4 ºC for 10 min at 16,000*g*. The supernatant was transferred to a clean polypropylene tube and stored at -80 ºC until analysis. For analysis, a volume of cold ACN was added to the serum. The sample was incubated in an ice bath for 10 min and centrifuged at 4 ºC for 10 min at 16,000*g*. The resulting organic layer was filtered through a polytetrafluoroethylene filter (0.2 µM, 13 mm diameter, Fisher Scientific) and 20 µL of the sample were analysed by LC-MS/MS (CAI Espectrometría de Masas, UCM). Separation was performed using a Phenomenex Gemini 5 µm C18 110A 150 x 2 mm column (run time 8 min; flow 0.4 mL/min; gradient: 3 min 5% to 35% B; 5 min 35% to 100% B; 8 min 5% B; phase A: water with formic acid 0.1%; phase B: ACN). The entire eluent was directly introduced to an electrospray ionization source operating in the positive ion mode in a Shimadzu LCMS8030 triple quadrupole mass spectrometer coupled to UHPLC with an oven temperature of 31.5 ºC. The mass spectrometer ion optics were set in the multiple reaction monitoring mode and the transition selected for quantification was set in the molecular weight range.

4.2.7. Pretreatment for proteomic experiments

Sorted $CD34^+$ cells were plated in 6-well plates at a density of 1 x 10^6 per well. Then, test compound or equivalent volume of DMSO was added. After 24 h, the cells were harvested, washed with PBS and lysed with ice-cold RIPA buffer (50 nM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal) containing protease and phosphatase inhibitors (Roche and Sigma-Aldrich, respectively). Lysates were clarified by centrifugation at 14000*g* for 5 min at 4 ºC and stored at -80 ºC until use. The digestion of peptides, labelling and LC-MS/MS analysis are in course in collaboration with Dr. Alberto Paradela of Centro Nacional de Biotecnología (CNB) in Madrid.

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5. REFERENCES

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