

ESTROGEN-RELATED RECEPTOR ALPHA (ERR α) MODULATORS5 **BACKGROUND TO THE INVENTION**

Estrogen-related receptor alpha (ERR α) is a 45.5 kilodalton (kDa), 423 amino acid residue protein which belongs to the nuclear receptor (NR) superfamily. This nuclear receptor family comprises 48 genes, coding for DNA-binding transcription factors that are involved in the regulation of diverse function including inter alia homeostasis, reproduction,
10 development and metabolism. The ERR family - the NR3B subgroup - consists of ERR α , ERR- β , and ERR- γ ; to date, no endogenous ligands have been identified for any of the ERR isoforms and therefore they are considered orphan receptors.

In Bookout et al. *Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network*, Cell.
15 126:789–99 (2006), organism-wide expression profiling of the three ERR isoforms determined that ERR α is widely distributed, with significant protein expression in most adult tissues. Knockout studies of the ERR family members have revealed that each receptor has tissue- and function-specific metabolic phenotypes that are important for adaptation to energy stress at the whole body level. Knockout studies have also indicated limited *in vivo* compensation among the ERR family members. The disclosure of *inter alia* Tremblay et al. *The NR3B subgroup: an overview*, Nuclear Receptor
20 Signaling, 5:e009 (2007) may be noted in this context.

Genomic studies have indicated that ERR α regulates large numbers of genes. The following references are instructive in this regard: Puigserver et al. *A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis*, Cell.
92(6):829–839 (1998); Yoon et al. *Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1*,
25 Nature 413(6852):131–138 (2001); Huss et al. *Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor α signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle*, Mol. Cell Biol. 24(20):9079–9091 (2004); and, Mootha et al. *ERR α and Gabpa/b specify PGC-1 α -dependent oxidative phosphorylation gene expression that is altered in diabetic muscle*, Proc. Natl. Acad. Sci. USA, 101(17):6570–6575 (2004).

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These references support a physiological model of ERR α function in regulating energy metabolism and, in particular, in the transcriptional regulation of genes required for mitochondrial biogenesis, the tricarboxylic acid cycle, oxidative phosphorylation, fatty acid oxidation and lipid metabolism. In particular, ERR α induces the expression of Nuclear Respiratory Factor 1 (NRF1), GA-binding protein alpha (GABP α), and Peroxisome Proliferator-activated Receptor alpha

(PPAR α). The nuclear receptor coactivators Peroxisome Proliferator-activated Receptor gamma coactivator 1-alpha (PGC-1 α), PGC-1 β and Peroxisome Proliferator-activated Receptor gamma Coactivator-related protein 1 (PPRC-1) are implicated in the regulation of these genes and in the auto-regulation of the expression of ERR α . PGC-1 α is expressed at low basal levels but is induced by fasting and other metabolic stresses. PGC-1 β , a related coactivator, has similar
5 functions, but its expression may not be regulated as acutely by variations in energy demand. Conversely, co-repressors that bind to ERRs, such as co-repressor nuclear Receptor Interacting Protein 140 (RIP140), compete with ERR co-activators to negatively regulate ERR-dependent gene expression.

The pleiotropic effect of ERR α activity on energy metabolism has interested the present inventors in the possibility that
10 it should be a target for the discovery of new therapies for diseases in which metabolic disturbances or modifications play a central role, such as type-2 diabetes, progressive heart failure, osteoporosis and cancer. Of particular interest is ERR α as a novel target for tumor therapy, through effects on the regulation of tumor cell energy metabolism associated with energy stress within tumor microenvironments. And of specific interest, is ERR α as a novel target for therapeutic
15 treatment of cancers with stem-like properties - Cancer Stem Cells (CSC), Tumor Initiating Cells (TIC) and Circulating Tumor Cells (CTC) - that rely on mitochondrial respiration for their energy requirements.

The initiation and development of cancer, in particular, is known to be associated with major metabolic alterations and mitochondria play a key role in tumorigenesis. A common abnormality observed in many cancer types – termed the Warburg effect - is a shift in glucose metabolism from oxidative phosphorylation to aerobic glycolysis and is
20 characterized by a drastic increase in glucose consumption accompanied by an elevated rate of lactate excretion regardless of oxygen abundance: aerobic glycolysis meets the metabolic needs of highly proliferative cells, including providing sufficient energy and providing for the accumulation of precursors for anabolic reactions. LeBleu et al. *PGC-1 α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis*, Nat. Cell Biol. 16(10):992-1 (2014) demonstrated that tumor cells display metabolic plasticity to engage either glycolysis or
25 oxidative phosphorylation depending on the tumor environment and their proliferative or metastasizing phenotype during cancer progression. It is thus evident that the targeting of metastatic progenitors and resistant tumor cells should not only happen via the glycolytic route but also via the mitochondrial oxidative phosphorylation.

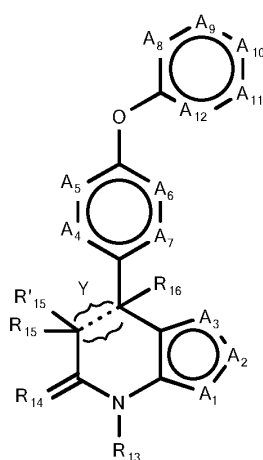
ERR α , together with PGC1 α/β , controls the regulation of genes encoding enzymes in the tricarboxylic acid (TCA) cycle
30 and oxidative phosphorylation. As discussed in Ariazi et al. *Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer*, Cancer Res. 62(22):6510-8 (2002), ERR α is expressed in a range of cancerous cells - including breast and prostate cancerous cells - and is associated with more invasive disease and a higher risk of recurrences in both these cancer types.

Chang et al. *The metabolic regulator ERR α , a downstream target of HER2/IGF-1R, as a therapeutic target in breast cancer*, Cancer Cell 20, 500-510 (2011) and Fujimoto et al. *Clinical implication of estrogen-related receptor (ERR) expression in ovarian cancers*, J. Steroid Biochem. Mol. Biol. 104, 301-304 (2007) document that ERR α is expressed in most cancers and that increased activity of this receptor is associated with a negative outcome in both breast and ovarian cancers. In the first of these references, it is confirmed that the transcription factor is involved in mitochondrial biogenesis and also in the regulation of oxidative phosphorylation. This latter point is considered important as resistance to the inhibition of KRAS pathway in pancreatic cancer, BRAF inhibitors in melanoma and oxaliplatin and 5-fluorouracil in colon cancer are also associated with a shift to oxidative metabolism.

The present inventors have therefore opined that inhibition of the activity of ERR α would enable a selective disruption of mitochondrial function in cancer, in particular in cancers of the aforementioned types. For this purpose, but equally for utility in the treatment of other ERR α mediated diseases and conditions, they have developed non-covalent, non-steroidal ERR α inverse agonists.

SUMMARY OF THE INVENTION

In accordance with a first aspect of the present invention, there is provided a compound according to Formula I



(Formula I)

or a pharmaceutically acceptable salt thereof, wherein

Y is a single carbon-carbon bond or a double carbon-carbon bond, with the proviso that when Y is a double

carbon-carbon bond, R'_{15} and R_{16} are not present;

one of the three positions A_1 – A_3 is either S or NR_A , the remaining two positions A_1 – A_3 are N or CR_1 , CR_2 , CR_3 , respectively;

R_A is H or methyl;

5 R_1 – R_3 are independently H, methyl, amino or halogen;

A_4 – A_7 are CR_4 , CR_5 , CR_6 and CR_7 , respectively;

A_8 – A_{12} are N or CR_8 , CR_9 , CR_{10} , CR_{11} and CR_{12} , respectively, with the proviso that no more than two of the five positions A_8 – A_{12} can be simultaneously N;

R_4 – R_7 are independently H, halogen, C(1-3)alkoxy, C(1-3)alkyl;

10 R_8 – R_{12} are independently H, halogen, C(1-3)alkoxy, C(1-4)alkyl, cyano, nitro, $-C(=O)OR_{17}$, hydroxyl, C(3-6)cycloalkyl, benzyl, phenyl, $-SF_5$, bicyclo[1.1.1]pentane;

or R_9 and either R_8 or R_{10} are fused and form an aromatic or non-aromatic five to seven membered ring containing two to seven carbon atoms and zero to three hetero atoms; with all groups optionally substituted with one or more methyl, halogen or hydroxyl;

15 R_{13} is H or methyl;

R_{14} is NH, O or S;

R_{15} and R'_{15} are independently H, halogen, C(1-4)alkyl, $-C(=O)OR_{17}$ or $-C(=O)NR_{17}R_{17}$;

R_{16} is H; and,

R_{17} is H, methyl or ethyl.

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In an embodiment, the invention relates to a compound according to Formula I in which A_1 is N, A_2 is NR_A and A_3 is CR_3 .

25 In another embodiment, the invention relates to a compound according to Formula I in which A_1 is N, A_2 is NH and A_3 is CH.

In another embodiment, the invention relates to a compound according to Formula I in which R_5 is C(1-3)alkoxy and R_4 , R_6 and R_7 are H.

30 In another embodiment, the invention relates to a compound according to Formula I in which R_5 is methoxy and R_4 , R_6 and R_7 are H.

In another embodiment, the invention relates to a compound according to Formula I in which A_8 – A_{12} are CR_8 , CR_9 , CR_{10} , CR_{11} and CR_{12} , respectively.

In another embodiment, the invention relates to a compound according to Formula I in which R₈-R₁₂ are independently H, C(1-4)alkyl, halogen, hydroxyl or C(1-3)alkoxy. For instance, R₈-R₁₂ may be independently H, C(1-4)alkyl or halogen. Good results have been obtained where R₉ and R₁₁ are independently C(1-4)alkyl, halogen, hydroxyl or C(1-3)alkoxy and R₈, R₁₀ and R₁₂ are H. And, of particular note, R₉ and R₁₁ are independently C(1-4)alkyl and R₈, R₁₀ and R₁₂ are H.

In another embodiment, the invention relates to a compound according to Formula I in which R₉ and R₁₁ are CF₃ and R₈, R₁₀ and R₁₂ are H.

In another embodiment, the invention relates to a compound according to Formula I in which R₁₃ is H.

In another embodiment, the invention relates to a compound according to Formula I in which R₁₄ is O.

In another embodiment, the invention relates to a compound according to Formula I in which R₁₅ and R'₁₅ are H.

In another embodiment, the invention relates to a compound according to Formula I in which Y is a single carbon-carbon bond.

The above embodiments, where they relate to a preferred form of different substituents of Formula (I), are not intended to be mutually exclusive of one another. Rather, combinations of these embodiments are envisaged within the scope of the present invention and, in certain circumstances, such combinations represent preferred structures for compounds of Formula I. In that regard, particular mention may be made of compounds according to Formula I in which: A₁ is N, A₂ is NH and A₃ is CH; A₄-A₇ are CR₄, CR₅, CR₆ and CR₇ respectively, wherein R₅ is C(1-3)alkoxy and R₄, R₆ and R₇ are H; A₈-A₁₂ are CR₈, CR₉, CR₁₀, CR₁₁ and CR₁₂, respectively, wherein R₉ and R₁₁ are independently C(1-4)alkyl, halogen, hydroxyl or C(1-3)alkoxy and R₈, R₁₀ and R₁₂ are H; R₁₃ is H; R₁₄ is O; R₁₅ and R'₁₅ are both H; Y is a single carbon-carbon bond; and, R₁₆ is H.

Mention may also be made of compounds according to Formula I in which: A₁ is N, A₂ is NH and A₃ is CH; A₄-A₇ are CR₄, CR₅, CR₆ and CR₇ respectively, wherein R₅ is C(1-3)alkoxy and R₄, R₆ and R₇ are H; A₈-A₁₂ are CR₈, CR₉, CR₁₀, CR₁₁ and CR₁₂, respectively, wherein R₉ and R₁₁ are independently C(1-4)alkyl and R₈, R₁₀ and R₁₂ are H; R₁₃ is H; R₁₄ is O; R₁₅ and R'₁₅ are both H; Y is a single carbon-carbon bond; and, R₁₆ is H.

DEFINITIONS

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

- 5 The terms "*comprising*", "*comprises*" and "*comprised of*" as used herein are synonymous with "*including*", "*includes*", "*containing*" or "*contains*", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. If used, the phrase "*consisting of*" is closed and excludes all additional elements. Further, the phrase "*consisting essentially of*" excludes additional material elements but allows the inclusion of non-material elements that do not substantially change the nature of the invention.
- 10 When amounts, concentrations, dimensions and other parameters are expressed in the form of a range, a preferable range, an upper limit value, a lower limit value or preferable upper and lower limit values, it should be understood that any ranges obtainable by combining any upper limit or preferable value with any lower limit or preferable value are also specifically disclosed, irrespective of whether the obtained ranges are clearly mentioned in the context.
- 15 The words "*preferred*", "*preferably*", "*desirably*" and "*particularly*" or synonyms thereof may be used frequently herein to refer to embodiments of the disclosure that may afford particular benefits, under certain circumstances. However, the recitation of one or more preferable, preferred, desirable or particular embodiments does not imply that other embodiments are not useful and is not intended to exclude those other embodiments from the scope of the disclosure.
- 20 As used throughout this application, the word "*may*" is used in a permissive sense – that is meaning to have the potential to - rather than in the mandatory sense.

As used herein "*room temperature*" is $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

- 25 Unless otherwise stated, the terms "*halo*" or "*halogen*" or "*halide*", as used herein by themselves or as part of another substituent, mean a fluorine, chlorine, bromine, or iodine atom. A preference for fluorine, chlorine or bromine is noted.

The term "*heteroatom*" as used herein represents nitrogen, oxygen or sulfur.

- 30 As used herein, "*C(1-n)alkyl*" group refers to a monovalent group that contains from 1 to n carbon atoms, that is a radical of an alkane and includes straight-chain and branched organic groups. As such, a "*C₁-C₃₀ alkyl*" group would refer to a monovalent group that contains from 1 to 30 carbon atoms, that is a radical of an alkane and includes straight-chain and branched organic groups. In the present invention, such alkyl groups may be unsubstituted or may be substituted

with the groups mentioned herein below. The halogenated derivatives of hydrocarbon radicals might, in particular, be mentioned as examples of suitable substituted alkyl groups.

5 The term "*C(1-4)alkyl*" as used herein means an alkyl group having 1-4 carbon atoms, i.e. methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl or *tert*-butyl. All carbon atoms may optionally be substituted with one or more halogen or hydroxyl.

10 The term "*C(1-3)alkyl*" as used herein means an alkyl group having 1-3 carbon atoms, i.e. methyl, ethyl, propyl or isopropyl. All carbon atoms may optionally be substituted with one or more halogen or hydroxyl.

10 The term "*C(3-6)cycloalkyl*" as used herein means a saturated cyclic hydrocarbon having 3-6 carbon atoms, i.e. cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl. All carbon atoms may optionally be substituted with one or more halogen or methyl.

15 The term "*C(1-3)alkoxy*" means an alkoxy group having 1-3 carbon atoms, the alkyl moiety being branched or unbranched. All carbon atoms are optionally substituted with one or more F or hydroxyl.

The term "*cyano*" as used herein, represents a group of formula -CN.

20 As used herein "*nitro group*" or "*nitro*" refers to -NO₂.

25 The term "*substituted*" means that one or more hydrogens on the designated atom(s) is/are replaced by a selection from the indicated group, provided that: the designated atom's normal valency under the existing circumstances is not exceeded; and, the substitution results in a stable compound. Combinations of substituents are also permissible only if such combinations result in stable compounds. The terms "*stable compound*" or "*stable structure*" refers to a compound or structure that is sufficiently robust to survive both isolation to a useful degree of purity from a reaction mixture and formulation into an efficacious therapeutic agent.

30 The term "*optionally substituted*" means optional substitution with the specified groups, radicals or moieties.

As used herein "*protecting group*" refers to a moiety attached to a functional group to prevent an undesired reaction. Preferably the protecting group may be easily removed after protection of the functional group is no longer required.

The compounds of Formula I may form salts, which are also within the scope of this invention. Reference to a compound of Formula I herein is understood to include reference to salts thereof, unless otherwise indicated.

5 The term "*pharmaceutically acceptable salt*" is used in accordance with its standard definition in the art to represent those salts which are, within the scope of medical judgment, suitable for use in contact with the tissues of humans and lower animals without, in particular, undue toxicity, irritation and / or allergic response: that use must be commensurate with a reasonable benefit to risk ratio. Pharmaceutically acceptable salts are well known in the art. They may either be obtained during the final isolation and purification of the compounds of the invention, or they may be obtained separately by reacting a free base function with: a suitable mineral acid, including but not limited to hydrochloric acid, phosphoric
10 acid or sulfuric acid; or, an organic acid, including but not limited to ascorbic acid, citric acid, tartaric acid, lactic acid, maleic acid, malonic acid, fumaric acid, glycolic acid, succinic acid, propionic acid, acetic acid or methanesulfonic acid. An acid function of compounds of the invention can be reacted with an organic or a mineral base, like sodium hydroxide, potassium hydroxide or lithium hydroxide. For completeness, organic bases include the common hydrocarbyl and heterocyclic amine salts, such as diethylamino, morpholine and piperidine salts, for example.

15 The compounds of Formula I may contain asymmetric or chiral centers and therefore exist in different stereoisomeric forms. It is intended that all stereoisomeric forms of the compounds of Formula I as well as mixtures thereof, including racemic mixtures, form part of the present invention. In particular, stereoisomeric forms of the compounds of Formula I which, following the Cahn-Ingold-Prelog system of nomenclature, are in the S configuration on the chiral center next to
20 the five-membered heterocyclic ring definitively form part of the present invention.

As will be understood by the skilled artisan, enantiomers can be separated by: converting the enantiomeric mixture into a diastereomeric mixture by reaction with an appropriate optically active compound, for instance a chiral auxiliary such as a chiral alcohol or Mosher's acid chloride; separating the diastereomers; and, converting – by hydrolysis for example
25 - the individual diastereomers to the corresponding pure enantiomers. Enantiomers can also be separated by use of chiral HPLC column.

It will be recognized further that various tautomers of compounds of Formula I may be possible: it is therefore intended that all tautomeric forms of compounds of Formula I form part of the invention. For completeness, as used herein, the
30 term "*tautomer*" refers to the migration of protons between adjacent single and double bonds. The tautomerization process is reversible: tautomers will generally reach an equilibrium state wherein the double bond is resonantly shared between two bond lengths.

The present invention also relates to a pharmaceutical composition comprising compounds or pharmaceutically acceptable salts thereof having the general Formula I in admixture with pharmaceutically acceptable excipients and optionally other therapeutically active agents. The excipients must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipients thereof.

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The invention further includes a compound of Formula I in combination with one or more other drug(s).

Compositions include, but are not limited to, those suitable for oral, sublingual, subcutaneous, intravenous, intramuscular, nasal, local, or rectal administration, all in unit dosage forms for administration. For oral administration, the active ingredient may be presented as discrete units, such as tablets, capsules, powders, granulates, solutions, suspensions and the like.

10

For parenteral administration, the pharmaceutical composition of the invention may be presented in unit-dose or multi-dose containers, such as injection liquids in predetermined amounts, presented for example in sealed vials and ampoules. The pharmaceutical composition may also be stored in a freeze dried (lyophilized) condition requiring only the addition of sterile liquid carrier – such as water - prior to use.

15

Mixed with such pharmaceutically acceptable auxiliaries, the active agent may be compressed into solid dosage units, such as pills, tablets, or be processed into capsules or suppositories. By means of pharmaceutically acceptable liquids, the active agent can be applied as a fluid composition - in the form of a solution, suspension or emulsion for instance – which may be included in an injection preparation or in a spray, such as a nasal spray.

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For making solid dosage units, the use of conventional additives such as fillers, colorants, polymeric binders and the like is contemplated. In general, any pharmaceutically acceptable additive which does not interfere with the function of the active compounds can be used. Suitable carriers with which the active agent of the invention can be administered as solid compositions include lactose, starch, cellulose derivatives and the like, or mixtures thereof, when used in suitable amounts. For parenteral administration, aqueous suspensions, isotonic saline solutions and sterile injectable solutions may be used, which suspensions or solutions may contain pharmaceutically acceptable dispersing agents and/or wetting agents, such as propylene glycol or butylene glycol.

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The invention further includes a pharmaceutical composition, as herein before described, in combination with packaging material suitable for said composition, said packaging material including instructions for the use of the composition for the purposes as hereinbefore described.

The exact dose and regimen of administration of the active ingredient, or a pharmaceutical composition thereof, may vary with the particular compound, the route of administration, and the age and condition of the individual subject to whom the medicament is to be administered.

- 5 In general parenteral administration requires lower dosages than other methods of administration which are more dependent upon absorption. That aside, a dosage for humans preferably contains from 0.0001 to 100 mg per kg body weight. The desired dose may be presented as one dose or as multiple sub-doses administered at appropriate intervals throughout the day.
- 10 The compounds according to the invention or a pharmaceutically acceptable salt thereof can be used as medicament in therapy.

- Another aspect of the invention resides in the use of compounds having the general Formula I or a pharmaceutically acceptable salt thereof for the therapeutic and / or prophylactic treatment of ERR α -mediated diseases or ERR α -mediated conditions. In particular, the invention provides for the use of compounds having the general Formula I or a pharmaceutically acceptable salt thereof for the treatment of ERR α -mediated cancer.
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- The compounds having the general Formula I or a pharmaceutically acceptable salt thereof can be used in therapies to treat at least one condition selected from: lung cancer; melanoma; endometrial cancer; and, acute myeloid leukemia.
- 20 Without intention to limit this aspect of the present invention, the compounds having the general Formula I or a pharmaceutically acceptable salt thereof may in particular be used in therapies to treat: superficial spreading melanoma; lentigo maligna; acral lentiginous melanoma; nodular melanoma; amelanotic melanoma; ocular melanoma; melanoma of the vulva; or, vaginal melanoma.

- In another aspect, the compounds having the general Formula I or a pharmaceutically acceptable salt thereof can be used in therapies to treat at least one condition selected from: breast cancer; bladder cancer; prostate cancer; pancreatic cancer; colorectal cancer; and, ovarian cancer.
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In another aspect, the compounds having the general Formula I or a pharmaceutically acceptable salt thereof can be used to treat Type II Diabetes Mellitus.

DETAILED DESCRIPTION OF THE INVENTION

As depicted in the Examples below, in certain exemplary embodiments compounds are prepared according to the following general procedures. It will be appreciated that, whilst the general methods depict the synthesis of certain compounds of the invention, the following general methods and other methods known to one skilled in the art, can be applied to all compounds and subclasses and species of each of these compounds, as described herein.

GENERAL METHODS OF PREPARATION

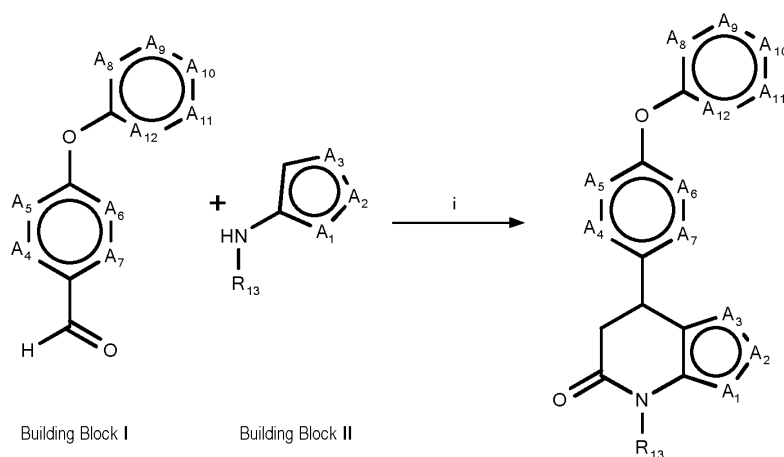
The compounds described herein, including compounds of general Formula I, Building Block I and Building Block II, are prepared by the reaction schemes depicted below. Furthermore, in the following schemes, where specific acids, bases, reagents, coupling agents, catalysts, solvents and the like are mentioned, it is understood that other suitable acid, bases, reagents, coupling agents, catalysts, solvents, etc. may be used and are included within the scope of the present invention. Modifications to the reaction conditions - for example, temperature, duration of the reaction or combinations thereof - are envisioned as part of the present invention.

The compounds obtained by using the general reaction sequences may be of insufficient purity. The compounds can be purified by using any of the methods for purification of organic compounds, for example, crystallization or silica gel or alumina column chromatography using different solvents in suitable ratios. All possible stereoisomers are envisioned within the scope of the invention.

Abbreviations for the materials employed in the Reaction Schemes and Examples are as follows:

AcOH: acetic acid; **ACN**: acetonitrile; **DAST**: (Diethylamino)sulfur trifluoride; **DCM**: dichloromethane; **DDQ**: 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone; **DIAD**: Diisopropyl azodicarboxylate; **DiBAL-H**: Diisobutylaluminium hydride; **DMAP**: 4-dimethylaminopyridine; **DMF**: *N,N*-dimethylformamide; **DMSO**: Dimethyl sulfoxide; **EDC**: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; **Et₂O**: di-ethyl ether; **EtOAc**: ethyl acetate; **EtOH**: Ethanol; **HPLC**: High-performance liquid chromatography; **MeOH**: Methanol; **PhSCu(I)**: phenylsulfanyl copper; **PPh₃**: Triphenylphosphine; **SFC**: Supercritical fluid chromatography; ***t*BuOK**: potassium *tert*-butoxide; ***t*BuONO**: *tert*-Butyl nitrite; **TEMPO**: 2,2,6,6-tetramethylpiperidinyloxy; **TFA**: trifluoroacetic acid; **THF**: tetrahydrofuran; **TOSMIC**: Tosylmethylisocyanide.

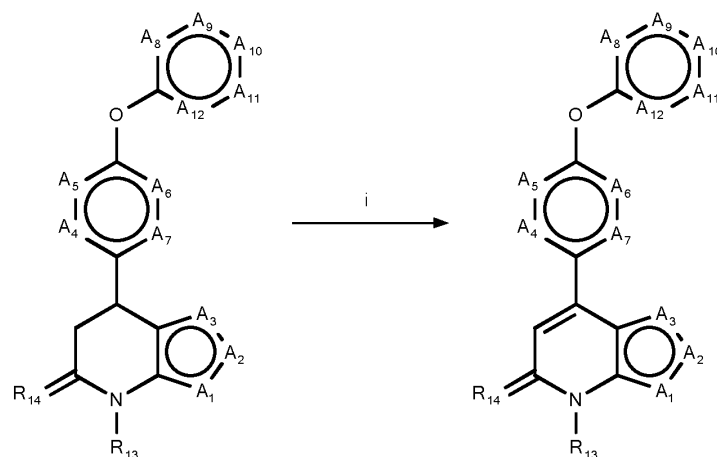
Chemical names are preferred IUPAC names, generated by using Marvin Sketch 17.24.1. If a chemical compound is referred to using both a chemical structure and a chemical name, and an ambiguity exists between the structure and the name, the structure predominates.

Scheme 1

Conditions: i) Meldrum's acid, MeOH

- Depicted in scheme 1 is the general synthesis of derivatives of the invention having Formula I, wherein R₁₄ is oxygen, R₁₅, R₁₅' and R₁₆ are H and Y is a single carbon-carbon bond. The derivatives can be prepared by methods known in the art of organic chemistry. Compounds of the invention can be obtained by a reaction between a benzaldehyde derivative of building block I, wherein A₄, A₅, A₆, A₇, A₈, A₉, A₁₀, A₁₁ and A₁₂, have the meaning as previously described, and a derivative of building block II, wherein R₁₃, A₁, A₂ and A₃ have the meaning as previously described, and Meldrum's acid.
- To obtain derivatives of Formula I wherein R₁₄ is sulphur, the derivatives of Formula I wherein R₁₄ is oxygen can be reacted with, for example, Lawesson's reagent.

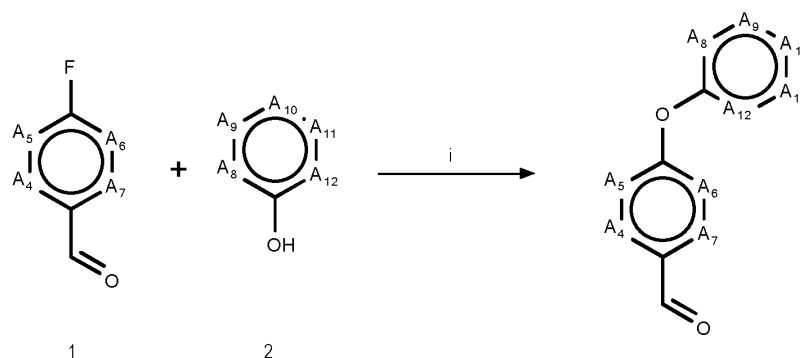
To obtain derivatives of Formula I wherein R₁₄ is nitrogen, the derivatives of Formula I wherein R₁₄ is sulphur can be reacted with, for example, ammonia in MeOH.

Scheme 1b

Conditions: i) DDQ, 1,4-dioxane

Scheme 1b depicts a general route for the preparation of Formula I analogs wherein Y is a double carbon-carbon bond,
 5 R₁₅ is H and R₁₃, R₁₄, A₁, A₂, A₃, A₄, A₅, A₆, A₇, A₈, A₉, A₁₀, A₁₁ and A₁₂ have the meaning as previously described.

Derivatives of Formula I, wherein Y is a single carbon-carbon bond and R₁₅, R₁₆ and R₁₇ are H, can be oxidized, using for example DDQ in an appropriate solvent, to obtain derivatives of Formula I, wherein Y is a double carbon-carbon bond.

Scheme 2

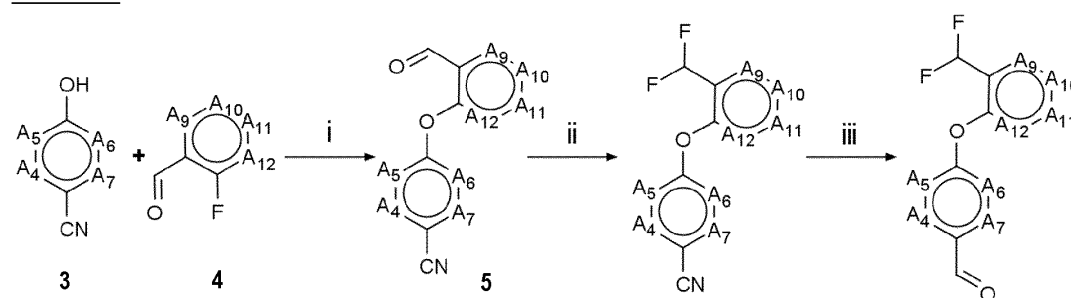
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Conditions: i) K₂CO₃, DMF

Scheme 2 depicts a general method for preparing benzaldehyde building block I derivatives wherein A₄, A₅, A₆, A₇, A₈, A₉, A₁₀, A₁₁ and A₁₂ have the meaning as previously described.

Aromatic substitution of 4-fluorobenzaldehyde **1** with phenol **2**, using K₂CO₃ in DMF at 110 °C, gives the building block I derivatives. For some substitutions on A₄ to A₁₂, it might be beneficial to use a 4-hydroxybenzaldehyde derivative with a fluorobenzene derivative to perform the aromatic substitution.

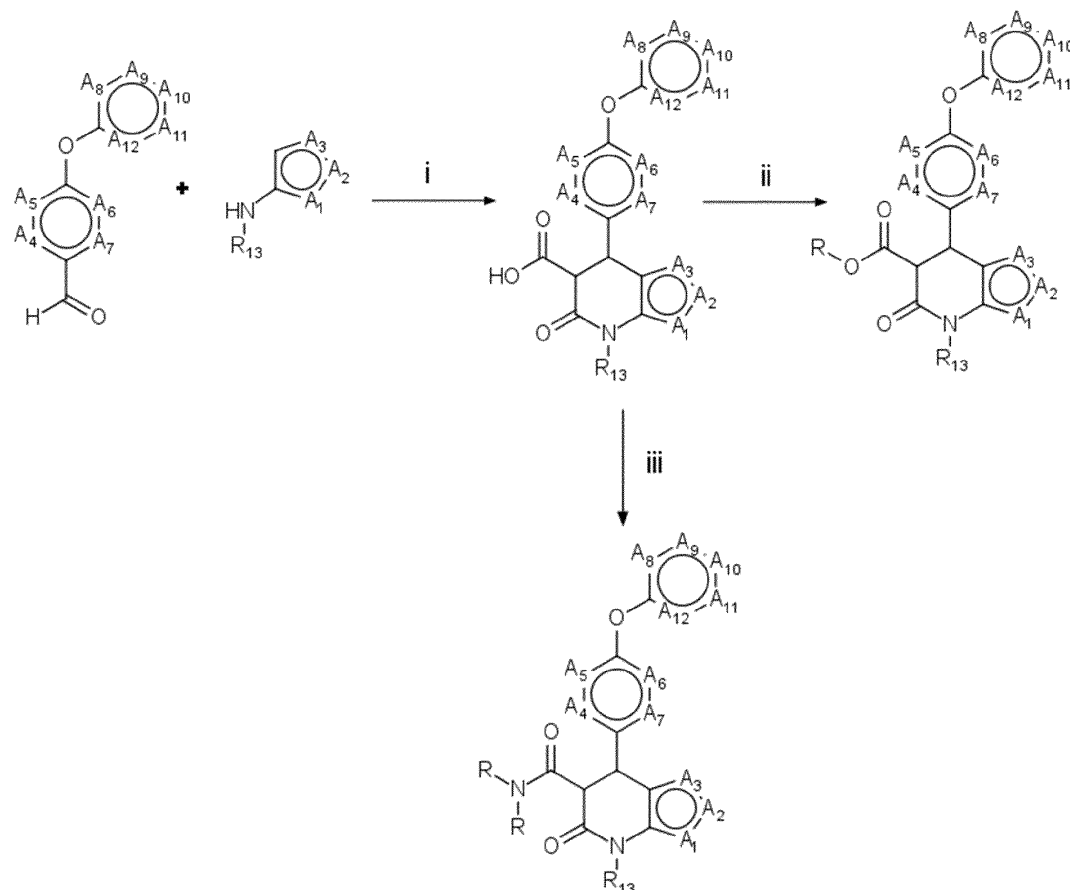
Scheme 2b



Conditions:

- i) K₂CO₃, DMF;
- ii) DAST, DCM;
- iii) DiBAL-H, toluene.

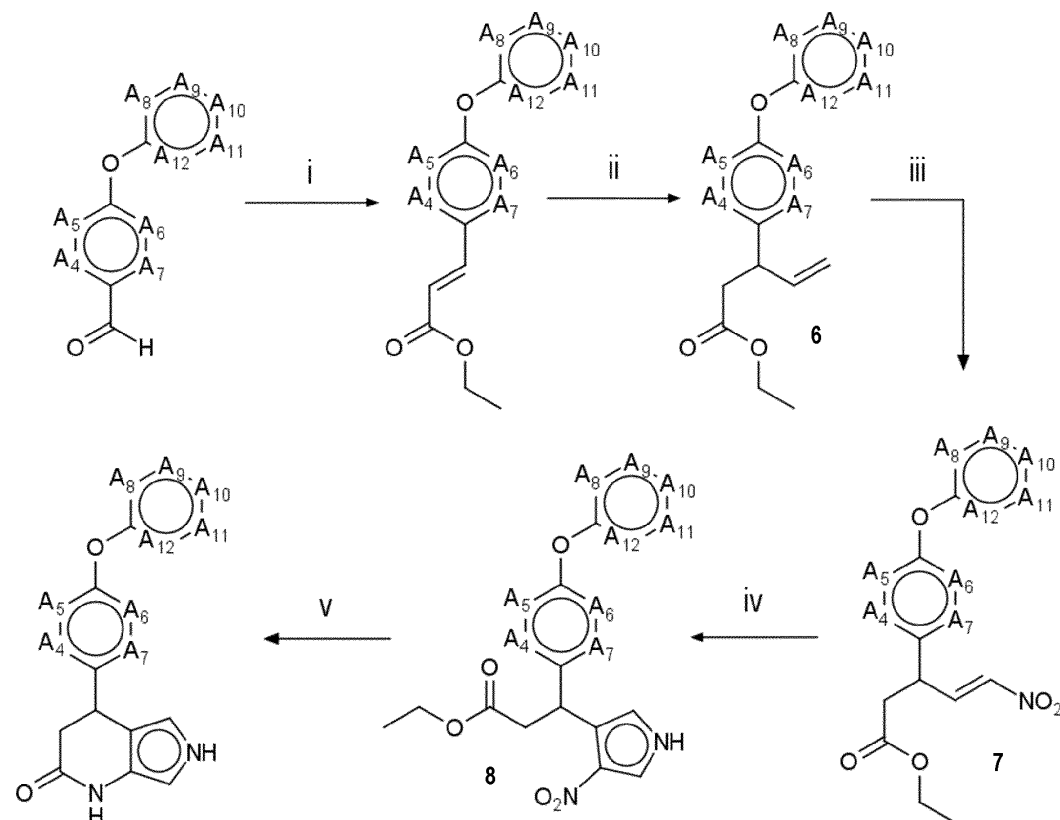
Scheme 2b depicts a general method for preparing benzaldehyde building block I derivatives, wherein A₈ is C-CF₂, and A₄, A₅, A₆, A₇, A₉, A₁₀, A₁₁ and A₁₂ have the meaning as previously described. Aromatic substitution of fluorobenzaldehyde **4** with 4-hydroxybenzonitrile **3** under basic condition, using for example K₂CO₃, gives the corresponding benzonitrile **5**. The aldehyde moiety can be converted to a CF₂ group using a fluorinating agent, for example DAST. Via subsequent reduction of the nitrile using, for example DiBAL-H in toluene, benzaldehyde building block I derivatives can be prepared. In scheme 2b this is exemplified for R₈, these conversion can also be applied for an aldehyde moiety in any of the positions R₄ to R₁₂. For some substitutions on A₄ to A₁₂, it might be beneficial to use a 4-fluorobenzonitrile derivative with a hydroxybenzaldehyde derivative to perform the aromatic substitution.

Scheme 3

Conditions:

- i) Meldrum's acid, MeOH;
- ii) ROH, DIAD, PPh₃, THF; and,
- iii) R₂NH, EDC, DMAP, DCM.

- 5 Scheme 3 depicts a general route for the preparation of Formula I analogs wherein Y is a single carbon-carbon bond, R₁₄ is oxygen, R₁₅ is COOH and each of R'₁₅ and R₁₆ is H. Compounds of the invention can be obtained by a reaction between a derivative of building block I, wherein A₄, A₅, A₆, A₇, A₈, A₉, A₁₀, A₁₁ and A₁₂ have the meaning as previously described, a derivative of building block II, wherein R₁₃, A₁, A₂, A₃ have the meaning as previously described, and Meldrum's acid at room temperature.
- 10 When R₁₅ is COOH, this carboxylic acid moiety can be functionalized towards an ester, using for example an alcohol, DIAD and PPh₃ in THF. Or it can be functionalized towards an amide, using for example a primary or secondary amine, EDC and DMAP in DCM.

Scheme 4

Conditions: i) $\text{CH}_3\text{CH}_2\text{OCOCH}_2\text{P}(\text{Ph})_3\text{Br}$, $t\text{BuOK}$, Et_2O ;

ii) CH_2CHMgBr , PhSCu(I) , THF;

iii) TEMPO, $t\text{BuONO}$, 1,4-dioxane;

iv) TSMIC, $t\text{BuOK}$, THF; and,

v) Zn, AcOH.

Scheme 4 illustrates a general route for the formation of Formula I analogs wherein A_1 and A_3 are CH, A_2 is NH, Y is a single carbon-carbon bond, R_{14} is oxygen, R_{13} is H, each of R_{15} , R'_{15} and R_{16} is H and A_4 , A_5 , A_6 , A_7 , A_8 , A_9 , A_{10} , A_{11} and A_{12} have the meaning as previously described.

- 10 Intermediate 6 can be obtained via a Wittig reaction of Building block I, using for example $\text{CH}_3\text{CH}_2\text{OCOCH}_2\text{P}(\text{Ph})_3\text{Br}$ and $t\text{BuOK}$ in Et_2O , followed by a reaction with, for instance, vinylmagnesium bromide and PhSCu(I) in THF. The subsequent introduction of the nitro group in the *E*-confirmation can be achieved by using, for example, TEMPO and $t\text{BuONO}$ in 1,4-dioxane. Pyrrole intermediate 8 was obtained via a [3+2] cycloaddition of intermediate 7, using for

instance TOSMIC and *t*BuOK in THF. The reduction of the nitro, followed by the ring closure to obtain the Formula I analog, was performed in a single step by using, for example, zinc dust in AcOH.

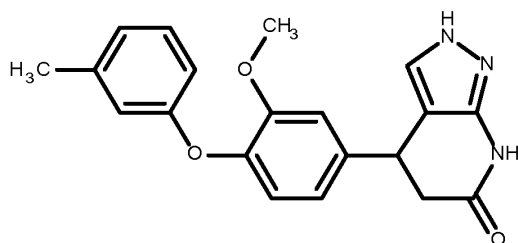
Examples

5

All building blocks used are commercially available, known or prepared according to methods known to those skilled in the art.

Examples 1-86

1: 4-[3-methoxy-4-(3-methylphenoxy)phenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



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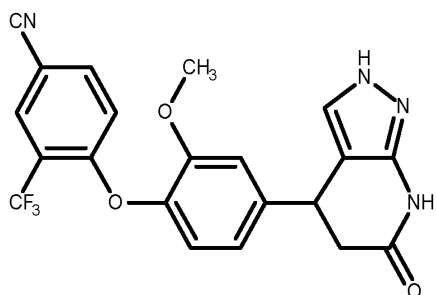
i) To a solution of meta-cresol (44 μ L) in DMF (4 mL) were added K_2CO_3 (175) and 4-fluoro-3-methoxybenzaldehyde (65 mg). The reaction mixture was heated to 110 $^{\circ}C$ and stirred overnight. After cooling to room temperature the solvent was removed under reduced pressure. The resulting oil was partitioned between DCM and water. The water layer was extracted twice more with DCM and the combined organic layers were dried over $MgSO_4$, filtered and concentrated. After reversed phased flash chromatography, using a gradient from 10% to 100% ACN in water, 3-methoxy-4-(3-methylphenoxy)benzaldehyde (66 mg) was obtained.

ii) The in the previous step obtained product (66 mg) was dissolved in 4 mL MeOH. Meldrum's acid (39 mg) and 3-aminopyrazole (23 mg) were added and the reaction was stirred 6 hours at 65 $^{\circ}C$. The reaction was cooled to room temperature, Celite was added and the solvent was evaporated. After reversed phase chromatography, using solids loading, 4-[3-methoxy-4-(3-methylphenoxy)phenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one (25 mg) was obtained. MS(ES^+) m/z 350.2 ($M+H$) $^+$

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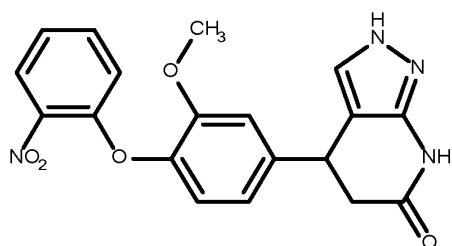
Following a procedure analogous to that described for example 1, using appropriate starting materials, the following compounds have been prepared.

2: 4-(2-methoxy-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)-3-(trifluoromethyl)benzonitrile



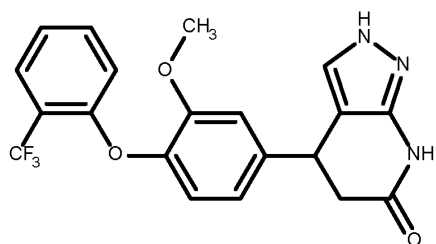
MS(ES⁺) *m/z* 429.2 (M+H)⁺

5 3: 4-[3-methoxy-4-(2-nitrophenoxy)phenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



MS(ES⁺) *m/z* 381.2 (M+H)⁺

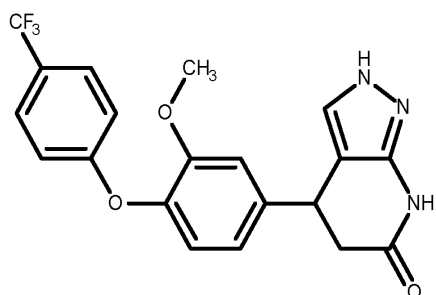
4: 4-{3-methoxy-4-[2-(trifluoromethyl)phenoxy]phenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



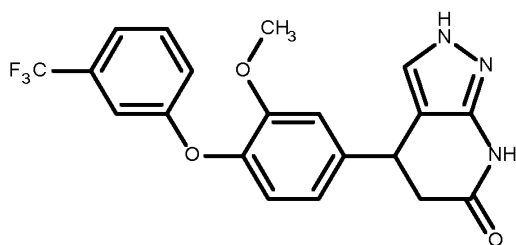
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MS(ES⁺) *m/z* 404.2 (M+H)⁺

5: 4-{3-methoxy-4-[4-(trifluoromethyl)phenoxy]phenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

MS(ES⁺) *m/z* 404.2 (M+H)⁺

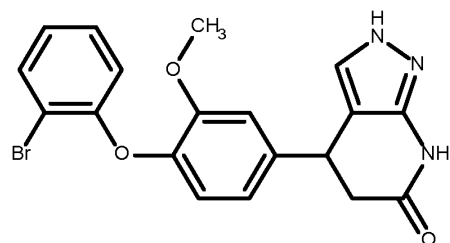
6: 4-[3-methoxy-4-[3-(trifluoromethyl)phenoxy]phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one



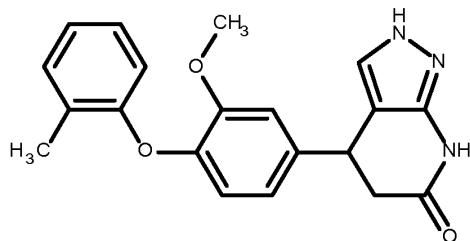
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MS(ES⁺) *m/z* 404.2 (M+H)⁺

7: 4-[4-(2-bromophenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

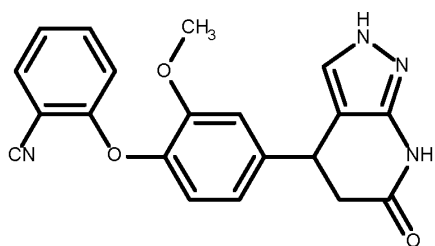
MS(ES⁺) *m/z* 414.2 (M+H)⁺

8: 4-[3-methoxy-4-(2-methylphenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one



MS(ES⁺) *m/z* 350.2 (M+H)⁺

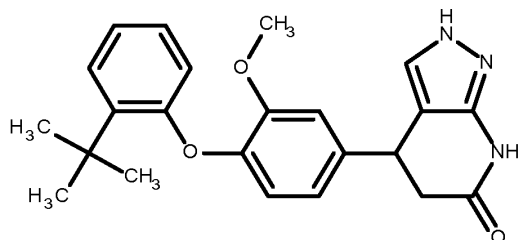
9: 2-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)benzonitrile



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MS(ES⁺) *m/z* 361.2 (M+H)⁺

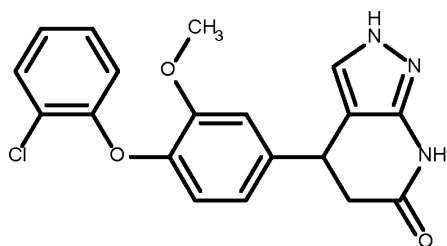
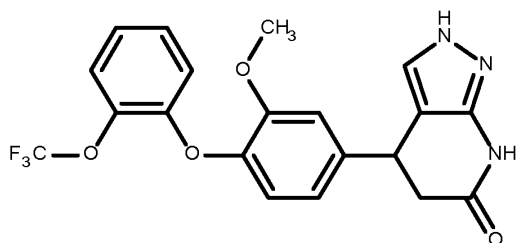
10: 4-[4-(2-*tert*-butylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one



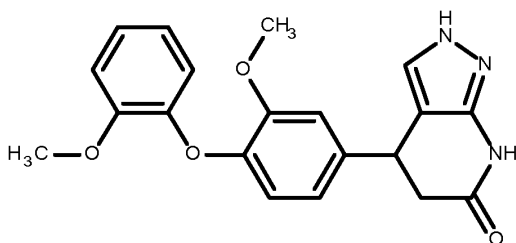
MS(ES⁺) *m/z* 392.2 (M+H)⁺

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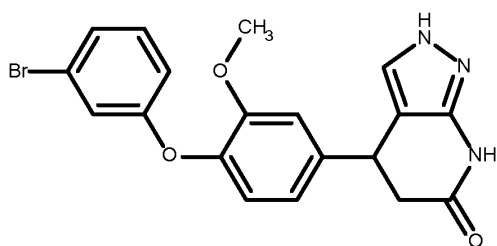
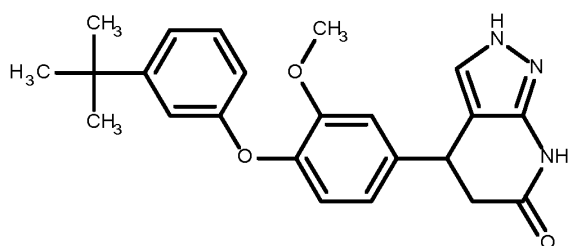
11: 4-[4-(2-chlorophenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

MS(ES⁺) *m/z* 370.2 (M+H)⁺**12:** 4-[3-methoxy-4-[2-(trifluoromethoxy)phenoxy]phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

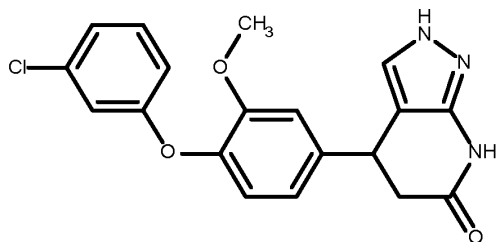
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MS(ES⁺) *m/z* 420.2 (M+H)⁺**13:** 4-[3-methoxy-4-(2-methoxyphenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 366.2 (M+H)⁺**14:** 4-[4-(3-bromophenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

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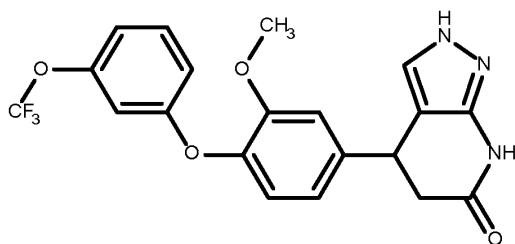
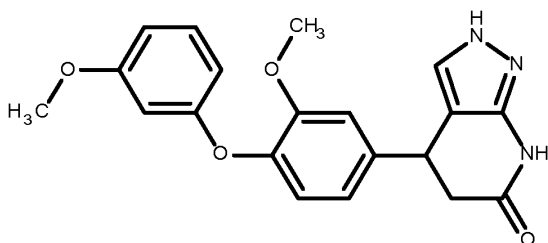
MS(ES⁺) *m/z* 414.2 (M+H)⁺**15:** 4-[4-(3-*tert*-butylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

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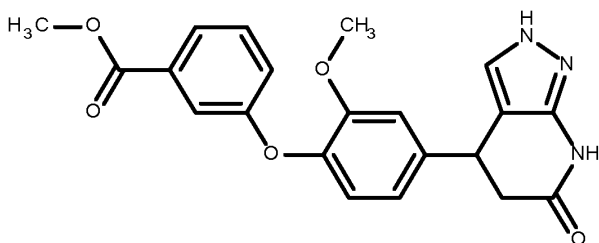
MS(ES⁺) *m/z* 392.2 (M+H)⁺**16:** 4-[4-(3-chlorophenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 370.2 (M+H)⁺

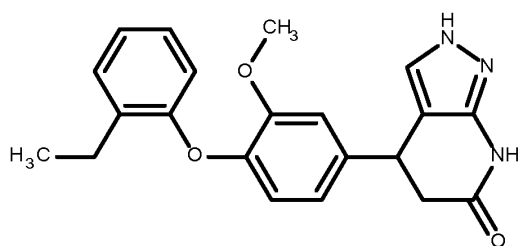
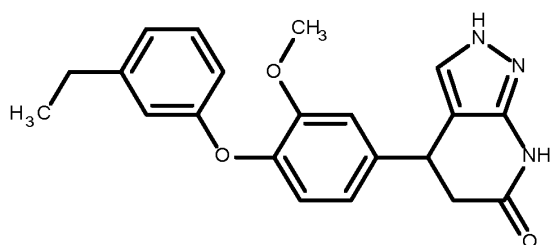
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17: 4-[3-methoxy-4-[3-(trifluoromethoxy)phenoxy]phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

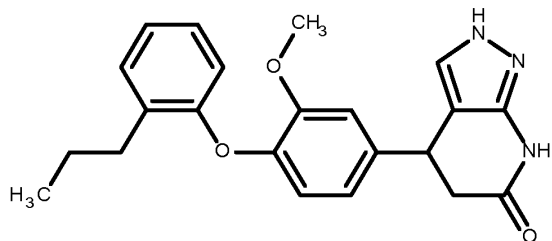
MS(ES⁺) *m/z* 420.2 (M+H)⁺**18:** 4-[3-methoxy-4-(3-methoxyphenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

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MS(ES⁺) *m/z* 366.2 (M+H)⁺**19:** methyl 3-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)benzoateMS(ES⁺) *m/z* 394.2 (M+H)⁺**20:** 4-[4-(2-ethylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

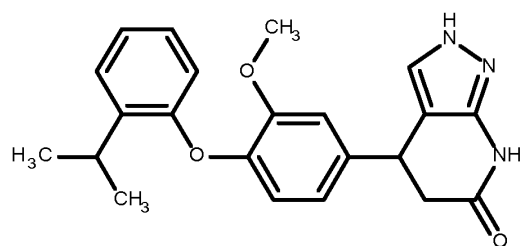
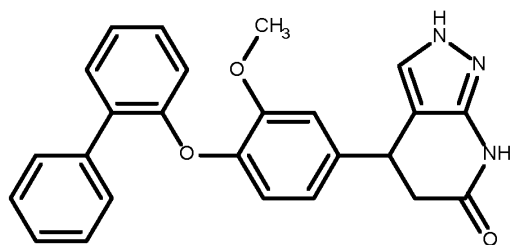
MS(ES⁺) *m/z* 364.2 (M+H)⁺**21:** 4-[4-(3-ethylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

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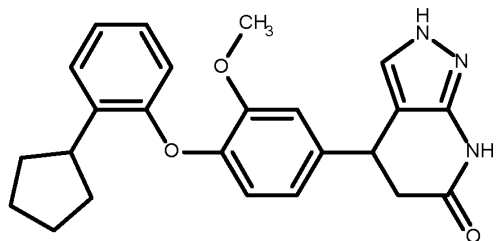
MS(ES⁺) *m/z* 364.2 (M+H)⁺**22:** 4-[3-methoxy-4-(2-propylphenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 378.2 (M+H)⁺

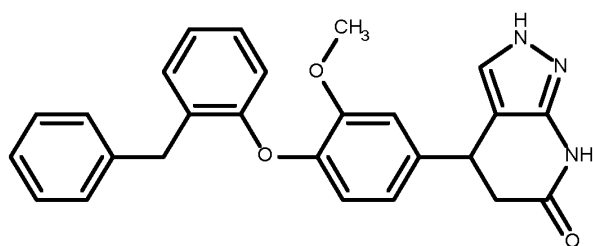
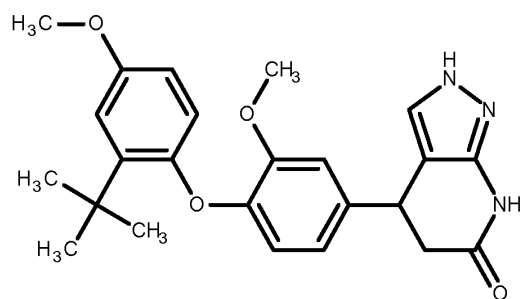
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23: 4-[3-methoxy-4-[2-(propan-2-yl)phenoxy]phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

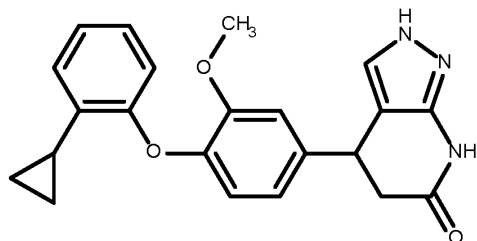
MS(ES⁺) *m/z* 378.2 (M+H)⁺**24:** 4-[3-methoxy-4-(2-phenylphenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

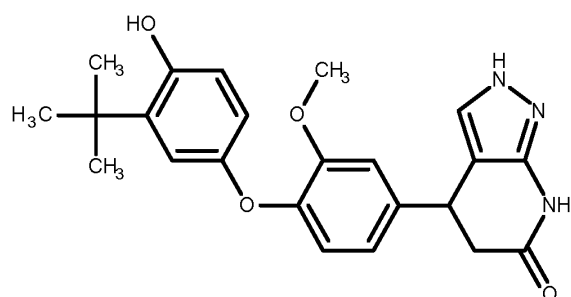
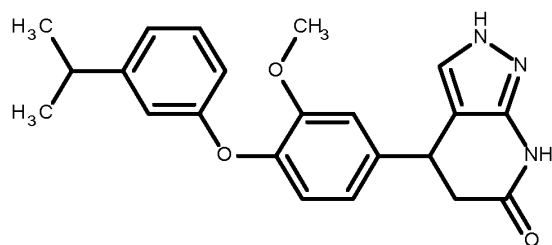
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MS(ES⁺) *m/z* 412.2 (M+H)⁺**25:** 4-[4-(2-cyclopentylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 404.2 (M+H)⁺**26:** 4-[4-(2-benzylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

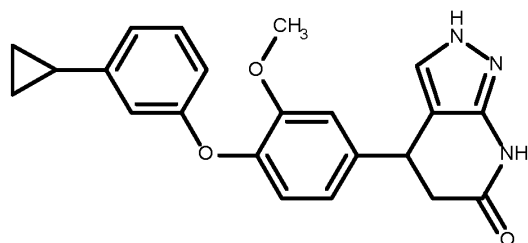
MS(ES⁺) *m/z* 426.2 (M+H)⁺**27:** 4-[4-(2-*tert*-butyl-4-methoxyphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

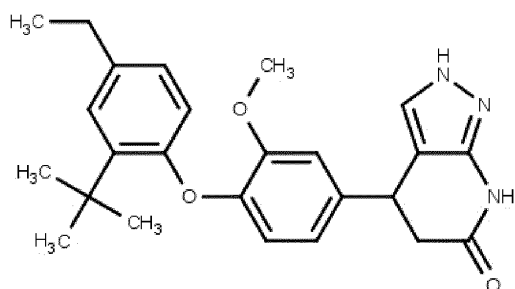
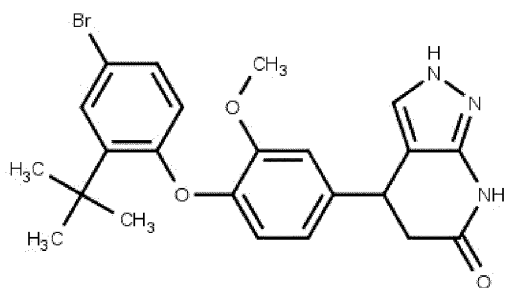
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MS(ES⁺) *m/z* 422.2 (M+H)⁺**28:** 4-[4-(2-cyclopropylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 376.2 (M+H)⁺**29:** 4-[4-(3-*tert*-butyl-4-hydroxyphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

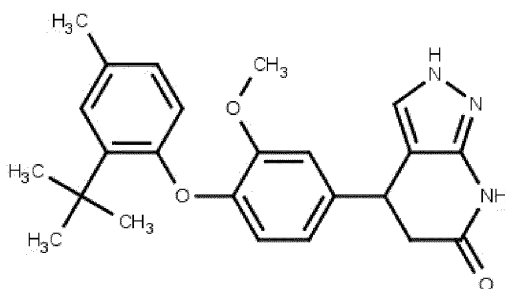
MS(ES⁺) *m/z* 408.2 (M+H)⁺**30:** 4-{3-methoxy-4-[3-(propan-2-yl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

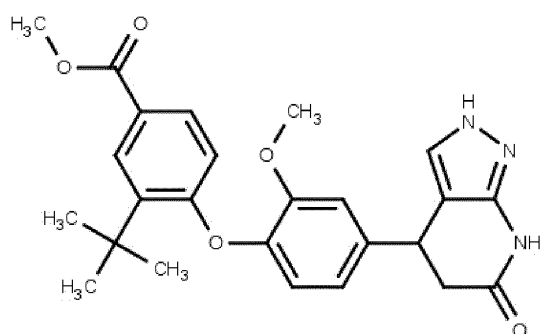
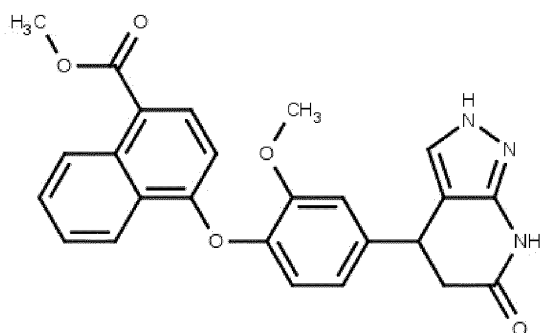
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MS(ES⁺) *m/z* 378.2 (M+H)⁺**31:** 4-[4-(3-cyclopropylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 376.2 (M+H)⁺**32:** 4-[4-(2-*tert*-butyl-4-ethylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

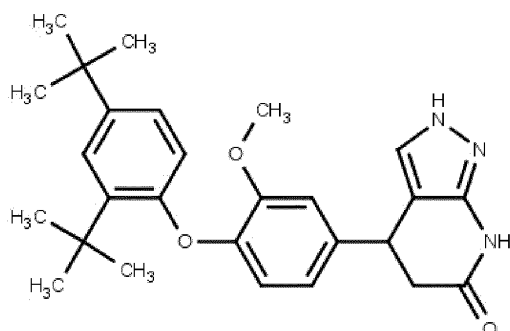
MS(ES⁺) *m/z* 420.2 (M+H)⁺**33:** 4-[4-(4-bromo-2-*tert*-butylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

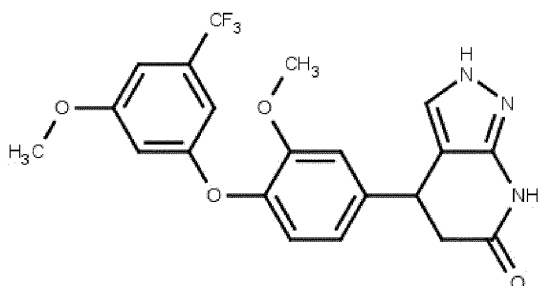
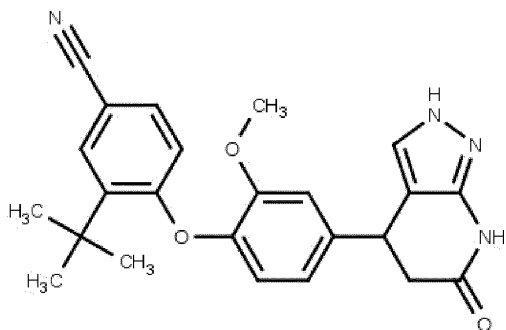
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MS(ES⁺) *m/z* 470.1 (M+H)⁺**34:** 4-[4-(2-*tert*-butyl-4-methylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 406.2 (M+H)⁺**35:** methyl 3-*tert*-butyl-4-(2-methoxy-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)benzoate

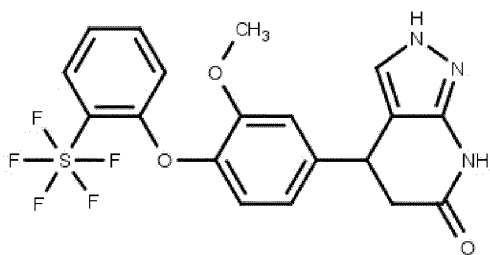
MS(ES⁺) *m/z* 450.2 (M+H)⁺**36:** methyl 4-(2-methoxy-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)naphthalene-1-carboxylate

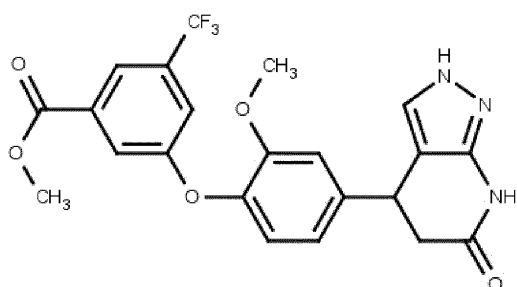
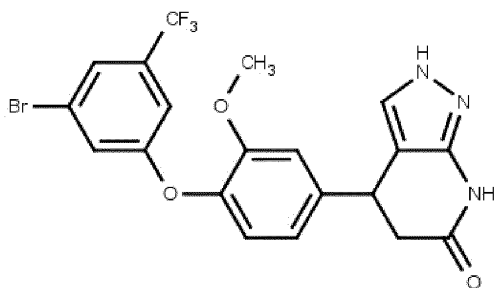
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MS(ES⁺) *m/z* 444.1 (M+H)⁺**37:** 4-[4-(2,4-di-*tert*-butylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 448.3 (M+H)⁺**38:** 4-{3-methoxy-4-[3-methoxy-5-(trifluoromethyl)phenoxy]phenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

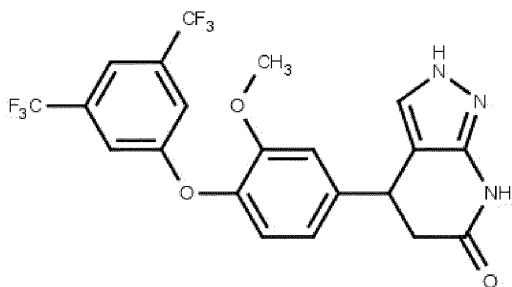
MS(ES⁺) *m/z* 434.1 (M+H)⁺**39:** 3-*tert*-butyl-4-(2-methoxy-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)benzonitrile

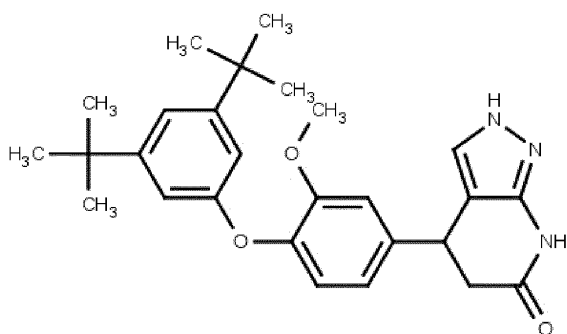
5

MS(ES⁺) *m/z* 417.2 (M+H)⁺**40:** 4-{3-methoxy-4-[2-(pentafluoro-λ⁶-sulfanyl)phenyl]phenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 462.1 (M+H)⁺**41:** methyl 3-(2-methoxy-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)-5-(trifluoromethyl)benzoate

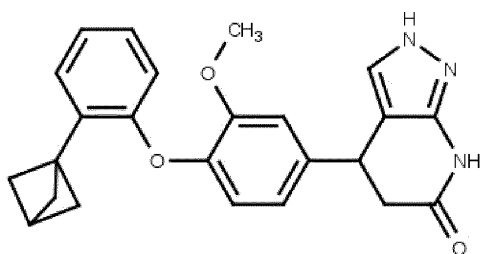
MS(ES⁺) *m/z* 462.1 (M+H)⁺**42:** 4-{4-[3-bromo-5-(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

5

MS(ES⁺) *m/z* 482.0 (M+H)⁺**43:** 4-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 472.1 (M+H)⁺**44:** 4-{4-[3,5-di-*tert*-butylphenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

MS(ES⁺) *m/z* 448.3 (M+H)⁺

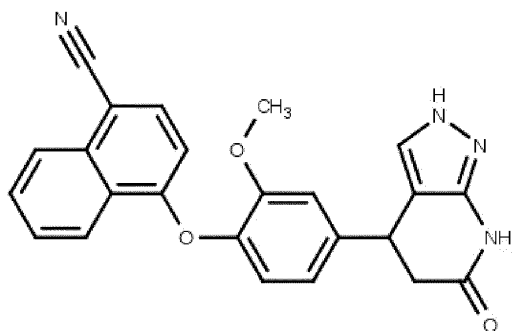
45: 4-[4-(2-{bicyclo[1.1.1]pentan-1-yl}phenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one



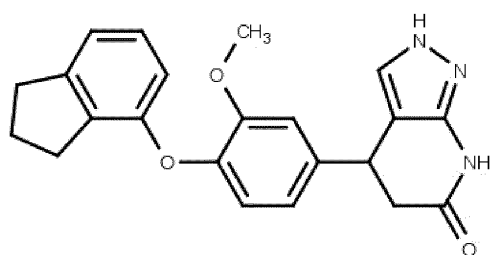
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MS(ES⁺) *m/z* 402.2 (M+H)⁺

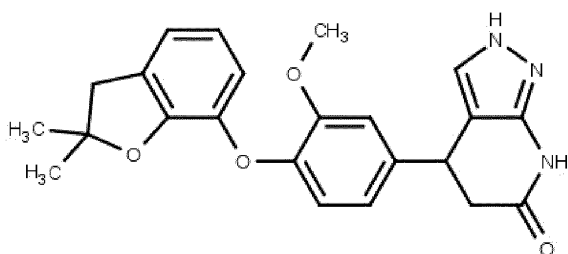
46: 4-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)naphthalene-1-carbonitrile

MS(ES⁺) *m/z* 411.1 (M+H)⁺

47: 4-[4-(2,3-dihydro-1H-inden-4-yloxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

MS(ES⁺) *m/z* 376.2 (M+H)⁺

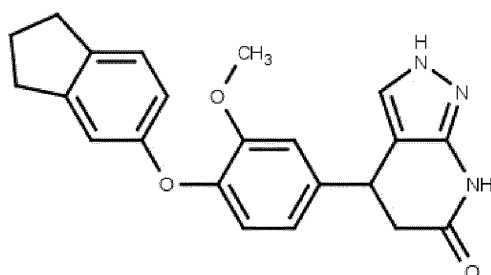
48: 4-{4-[(2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl)oxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one



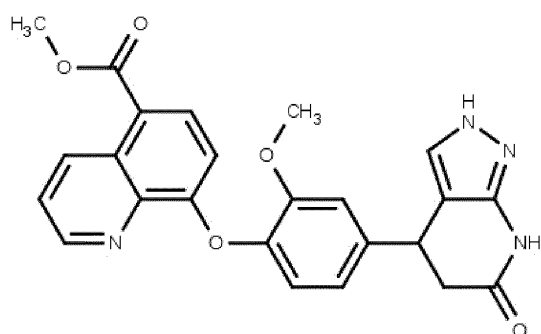
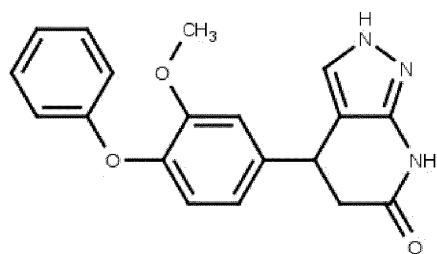
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MS(ES⁺) *m/z* 406.2 (M+H)⁺

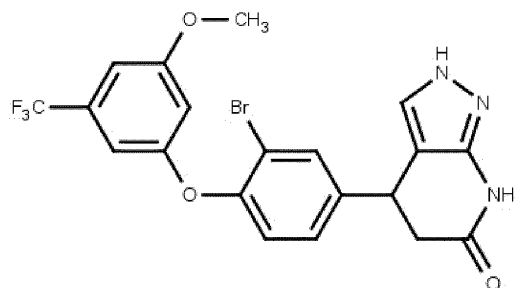
49: 4-[4-(2,3-dihydro-1H-inden-5-yloxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

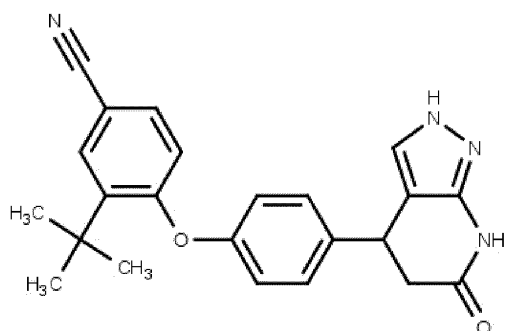
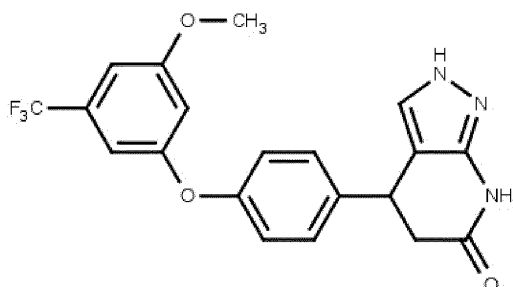
MS(ES⁺) *m/z* 376.2 (M+H)⁺

10 **50:** methyl 8-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)quinoline-5-carboxylate

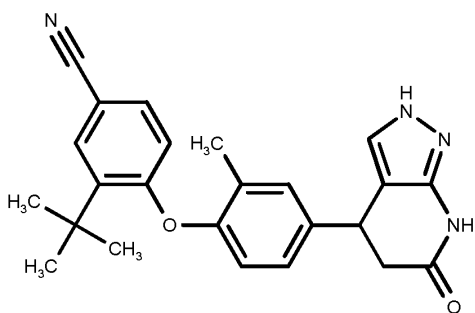
MS(ES⁺) *m/z* 445.1 (M+H)⁺**51:** 4-(3-methoxy-4-phenoxyphenyl)-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

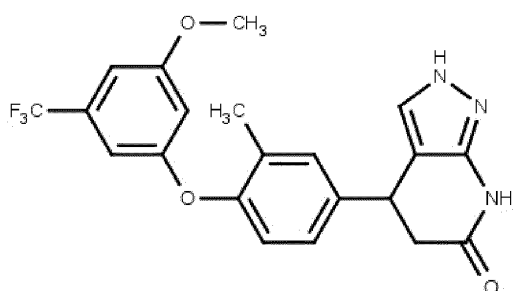
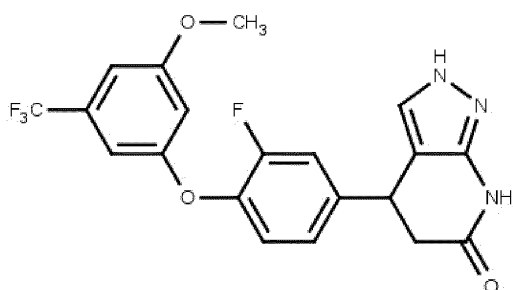
5

MS(ES⁺) *m/z* 336.1 (M+H)⁺**52:** 4-{3-bromo-4-[3-methoxy-5-(trifluoromethyl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 482.0 (M+H)⁺**53:** 3-*tert*-butyl-4-(4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)benzonitrile

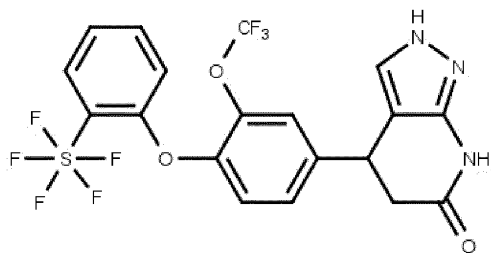
MS(ES⁺) *m/z* 387.2 (M+H)⁺**54:** 4-{4-[3-methoxy-5-(trifluoromethyl)phenoxy]phenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

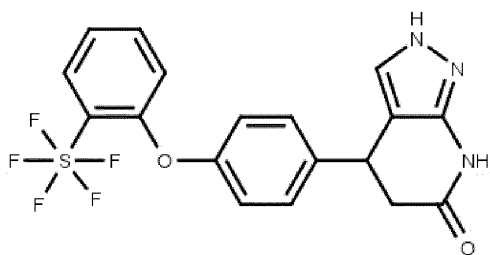
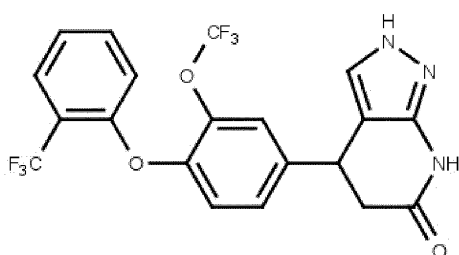
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MS(ES⁺) *m/z* 404.1 (M+H)⁺**55:** 3-*tert*-butyl-4-(2-methyl-4-{6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)benzonitrileMS(ES⁺) *m/z* 400.2 (M+H)⁺**56:** 4-{4-[3-methoxy-5-(trifluoromethyl)phenoxy]-3-methylphenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

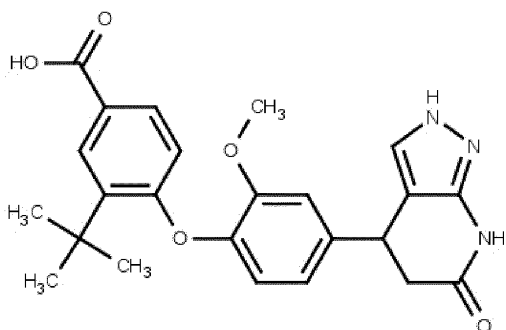
MS(ES⁺) *m/z* 418.1 (M+H)⁺**57:** 4-{3-fluoro-4-[3-methoxy-5-(trifluoromethyl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

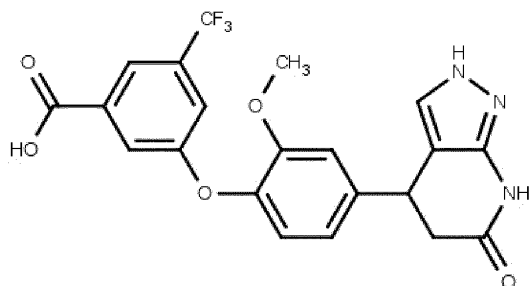
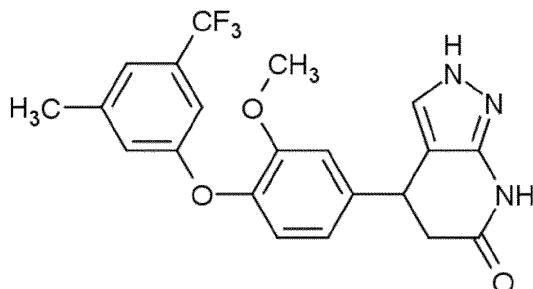
5

MS(ES⁺) *m/z* 422.1 (M+H)⁺**58:** 4-{4-[2-(pentafluoro-λ⁶-sulfanyl)phenoxy]-3-(trifluoromethoxy)phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-oneMS(ES⁺) *m/z* 516.1 (M+H)⁺10 **59:** 4-{4-[2-(pentafluoro-λ⁶-sulfanyl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

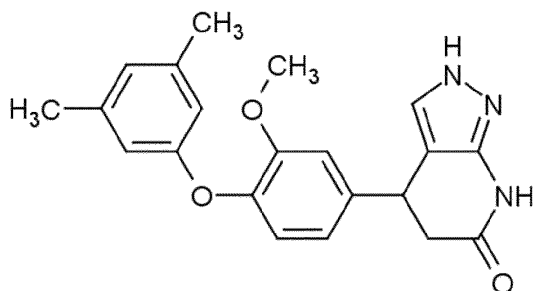
MS(ES⁺) *m/z* 432.1 (M+H)⁺**60:** 4-[3-(trifluoromethoxy)-4-(2-(trifluoromethyl)phenoxy)phenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one

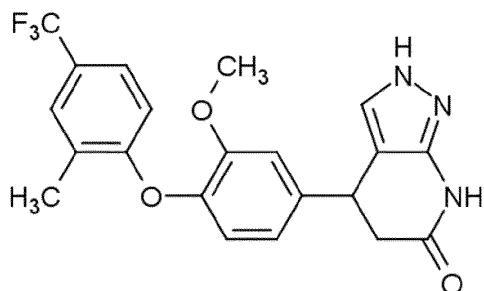
5

MS(ES⁺) *m/z* 458.1 (M+H)⁺**61:** 3-*tert*-butyl-4-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)benzoic acidMS(ES⁺) *m/z* 436.2 (M+H)⁺10 **62:** 3-(2-methoxy-4-{6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-4-yl}phenoxy)-5-(trifluoromethyl)benzoic acid

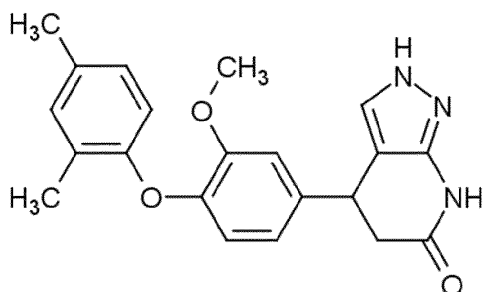
MS(ES⁺) *m/z* 448.1 (M+H)⁺**63:** 4-{3-methoxy-4-[3-methyl-5-(trifluoromethyl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

5

MS(ES⁺) *m/z* 418.4 (M+H)⁺**64:** 4-[4-(3,5-dimethylphenoxy)-3-methoxyphenyl]-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-oneMS(ES⁺) *m/z* 364.4 (M+H)⁺**65:** 4-{3-methoxy-4-[2-methyl-4-(trifluoromethyl)phenoxy]phenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-*b*]pyridin-6-one

MS(ES⁺) *m/z* 418.4 (M+H)⁺

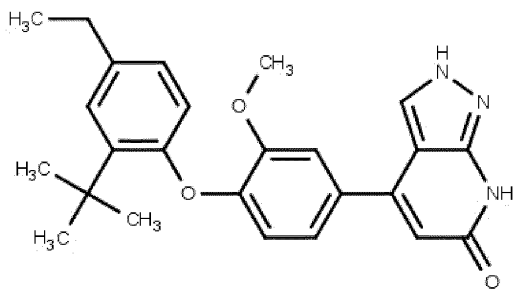
66: 4-[4-(2,4-dimethylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



5

MS(ES⁺) *m/z* 364.4 (M+H)⁺

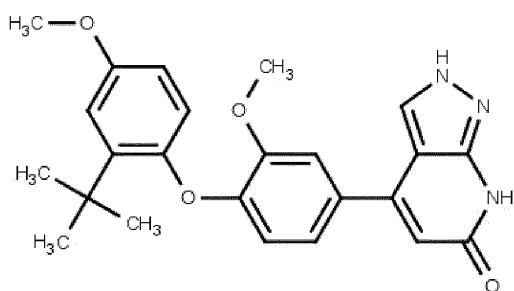
67: 4-[4-(2-*tert*-butyl-4-ethylphenoxy)-3-methoxyphenyl]-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



i) Example 32 (32 mg) was dissolved in 1.5 mL 1,4-dioxane and DDQ (17 mg) was added. The reaction was heated to 50 °C and stirred for 1 hour. The solvent was evaporated and sat. aq. NaHCO₃ was added to give a suspension. The suspension was sonicated and the solids were filtered off and washed with water three times. The solids were dried in the vacuum oven to afford 4-[4-(2-*tert*-butyl-4-ethylphenoxy)-3-methoxyphenyl]-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one (23 mg). MS(ES⁺) *m/z* 418.2 (M+H)⁺

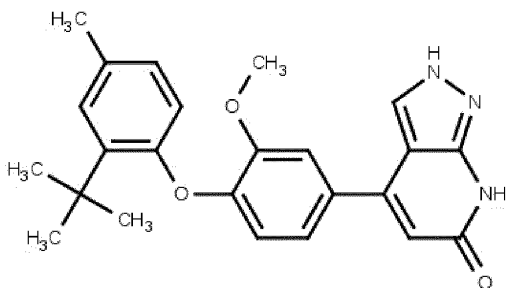
Following a procedure analogous to that described for **Example 67**, using appropriate starting materials, the following compounds have been prepared.

- 5 **68:** 4-[4-(2-*tert*-butyl-4-methoxyphenoxy)-3-methoxyphenyl]-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



MS(ES⁺) *m/z* 420.2 (M+H)⁺

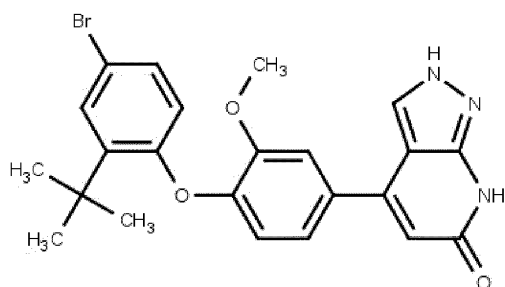
- 69:** 4-[4-(2-*tert*-butyl-4-methylphenoxy)-3-methoxyphenyl]-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



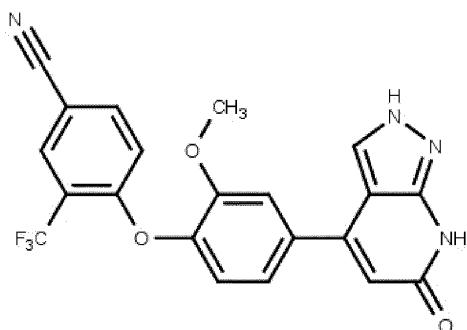
10

MS(ES⁺) *m/z* 404.2 (M+H)⁺

- 70:** 4-[4-(4-bromo-2-*tert*-butylphenoxy)-3-methoxyphenyl]-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

MS(ES⁺) *m/z* 468.1 (M+H)⁺

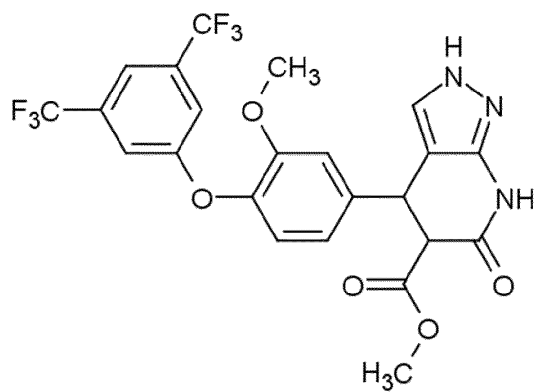
71: 4-(2-methoxy-4-{6-oxo-2*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-4-yl}phenoxy)-3-(trifluoromethyl)benzonitrile



5

MS(ES⁺) *m/z* 427.1 (M+H)⁺

72: methyl 4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-6-oxo-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylate



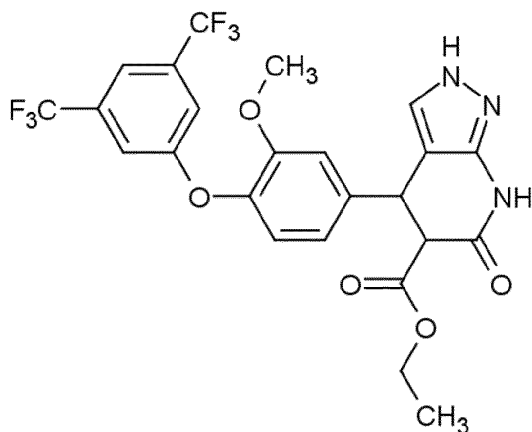
i) Following a procedure analogous to that described in **Example 1, step i)**, using appropriate starting materials, 4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxybenzaldehyde was obtained.

ii) The in the previous step obtained product (211 mg), Meldrum's acid (85 mg) and 3-aminopyrazole (48 mg) were dissolved in MeOH (5 mL). The reaction mixture was stirred for 3.5 hours at room temperature and the solvent was evaporated by a flow of nitrogen. This resulted in 4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxylic acid (200 mg).

iv) DIAD (23 μ L) was added dropwise under a nitrogen atmosphere to a solution of PPh₃ (31.4 mg), MeOH (40 μ L) and the in the previous step obtained product (50 mg) in anhydrous THF (1 mL). The reaction was stirred overnight at room temperature. Water was added and the product was extracted with DCM three times. The combined organic layers were dried over MgSO₄ and concentrated. The crude product was purified by reversed phase chromatography, using a gradient of 40 to 75% ACN in water (containing 0.1% TFA), to obtain methyl 4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxylate (16.6 mg). MS(ES⁺) *m/z* 530.4 (M+H)⁺

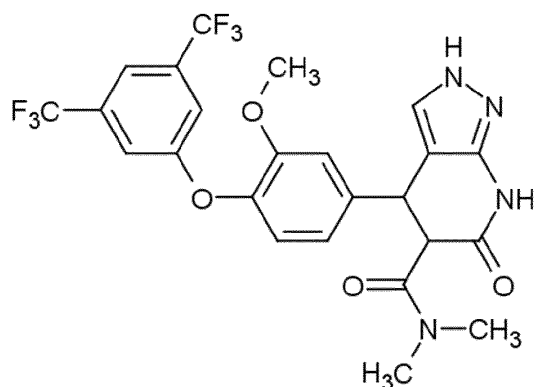
Following a procedure analogous to that described for **Example 72**, using appropriate starting materials, the following compound has been prepared.

73: ethyl 4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxylate



MS(ES⁺) *m/z* 544.4 (M+H)⁺

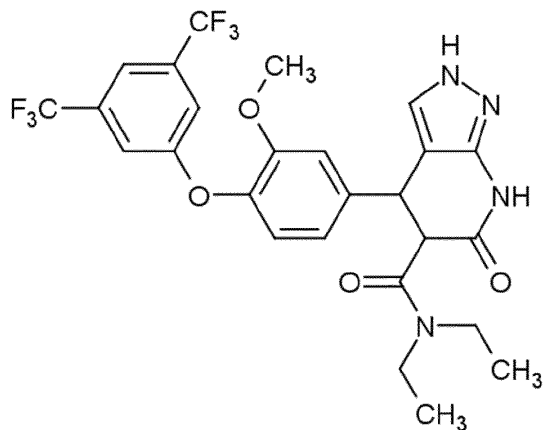
74: 4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-N,N-dimethyl-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxamide



- i) The product obtained in **Example 72, step ii)** (50 mg), DMAP (20 mg) and dimethylamine HCl (12 mg) were dissolved in DCM (1 mL). EDC (30 mg) was added and the reaction was stirred at room temperature overnight. Water was added and the product was extracted with DCM three times. The combined organic layers were dried over MgSO_4 and concentrated. The crude product was purified by reversed phase chromatography, using a gradient of 40 to 75% ACN in water (containing 0.1% TFA), to obtain 4-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-N,N-dimethyl-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxamide (9.3 mg). MS(ES^+) m/z 543.4 ($\text{M}+\text{H}$)⁺

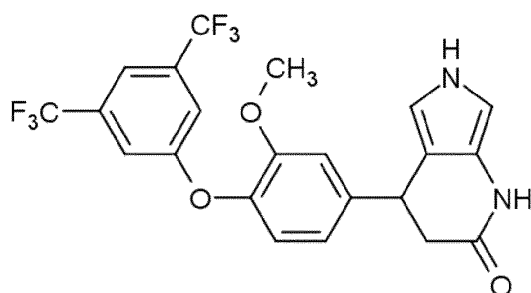
Following a procedure analogous to that described for **Example 74**, using appropriate starting materials, the following compound has been prepared.

- 10 **75:** 4-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-N,N-diethyl-6-oxo-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridine-5-carboxamide



MS(ES^+) m/z 571.5 ($\text{M}+\text{H}$)⁺

- 15 **76:** 4-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-1H,2H,3H,4H,6H-pyrrolo[3,4-b]pyridine-2-one



i) Following a procedure analogous to that described in **Example 1, step i)**, using appropriate starting materials, 4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxybenzaldehyde was obtained.

5 ii) Ethoxycarbonylmethyl)triphenylphosphonium bromide (3.54 g) was added to a suspension of *t*BuOK (1.03 g) in Et₂O (30 mL). The resulting mixture stirred at 50 °C for 30 minutes and then cooled to room temperature. The in the previous step obtained compound (1.50 g) dissolved in Et₂O (10 mL) was added to the reaction mixture. The reaction was stirred overnight, then quenched with water and the aqueous layer was extracted with Et₂O three times. The combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified on silica column, using a gradient of 0 to 20% EtOAc in heptane, to obtain ethyl (E)-3-{4-[3,5-
 10 bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}prop-2-enoate (1.42 g).

15 iii) Vinylmagnesium bromide (1.0 M in THF, 9.67 mL) was added dropwise to a suspension of phenylsulfanylcopper (558 mg) in anhydrous THF (15 mL) at -40 °C under a nitrogen atmosphere. The reaction was allowed to reach -25 °C and again cooled down to -40 °C. The in the previous step obtained compound (1.4 g) in anhydrous THF (15 mL) was added dropwise and the reaction was stirred for 30 minutes at -40 °C. The reaction was quenched by pouring the mixture into sat. aq. NH₄Cl. The obtained solids were filtered off and the filtrate was extracted with EtOAc twice. The combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified on silica column, using a gradient of 0 to 30% EtOAc in heptane, to obtain ethyl 3-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}pent-4-enoate (279 mg).

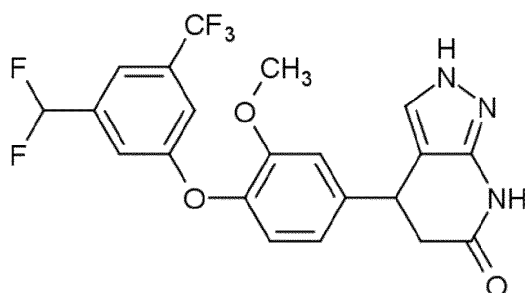
20 iv) The in the previous step obtained product (275 mg), TEMPO (38 mg) and *t*BuONO (157 μ L) were dissolved in 1,4-dioxane (5 mL), heated to 80 °C and stirred overnight. Water was added to the reaction and the aqueous layer was extracted with DCM three times. The combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified on silica column, using a gradient of 0 to 30% EtOAc in heptane, to obtain ethyl (E)-3-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-5-nitropent-4-enoate (95 mg).

25 v) TOSMIC (37 mg) was added to a solution of *t*BuOK (42 mg) in anhydrous THF (1 mL) at -78 °C and stirred for 10 minutes. The compound obtained from the previous step (95 mg) was dissolved in anhydrous THF (4 mL) and added dropwise to the reaction mixture. After 10 minutes stirring at -78 °C the reaction was poured into sat. aq. NH₄Cl and

the aqueous layer was extracted with EtOAc three times. The combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified on silica column, using a gradient of 0 to 70% EtOAc in heptane, to obtain ethyl 3-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-3-(4-nitro-1*H*-pyrrol-3-yl)propanoate (48 mg).

- 5 **vi)** To a solution of the in the previous step obtained compound (45 mg) in AcOH (1.5 mL) was added zinc dust (54 mg). The reaction was heated to 50 °C and stirred for 30 minutes. The reaction mixture was filtered over celite and the filter cake was rinsed with EtOAc. The filtrate was washed with sat. aq. NaHCO₃ and water and then dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified by reversed phase chromatography, using a gradient of 40 to 90% ACN in water, to obtain 4-{4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl}-1*H*,2*H*,3*H*,4*H*,6*H*-pyrrolo[3,4-*b*]pyridin-2-one (8.7 mg). MS(ES⁺) *m/z* 471.4 (M+H)⁺
- 10

77: 4-{4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one



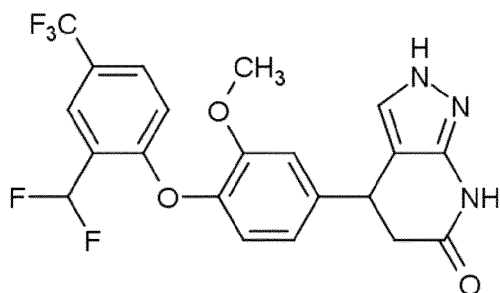
- i) 4-Fluoro-3-methoxy-benzonitrile (250 mg), 3-hydroxy-5-(trifluoromethyl)benzaldehyde (0.23 mL) and K₂CO₃ (457 mg) suspended in DMF (10mL). The reaction was heated to 90°C and stirred overnight. The reaction was cooled to room temperature and partitioned between water and EtOAc. The water layer was extracted twice more with EtOAc and the combined organic layers were washed with brine, dried over MgSO₄ and concentrated. The crude product was purified by reversed phase chromatography, using a gradient of 10 to 100% ACN in water, to obtain 4-[3-formyl-5-(trifluoromethyl)phenoxy]-3-methoxy-benzonitrile (46 mg).
- 15
- ii) The in the previous step obtained product (96 mg) was dissolved in DCM (6 mL) under a nitrogen atmosphere. DAST (0.14 mL) was added dropwise and the reaction was stirred overnight at room temperature. The reaction was placed on a water bath and sat. aq. NaHCO₃ (15 mL) was added. The mixture was stirred vigorously for 10 min. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over MgSO₄ and concentrated to afford 4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxy-benzonitrile (102 mg).
- 20
- iii) The in the previous step obtained compound (101 mg) was dissolved in toluene (4.5 mL) under a nitrogen atmosphere and cooled to 0 °C. A DiBAL-H solution 1.2M in toluene (0.37 mL) was added dropwise and the reaction
- 25

was stirred for 1.5 hours. Aqueous HCl 6N (1.5 mL) was added and the mixture was stirred until the bubbling stopped. The mixture was warmed to room temperature and partitioned between EtOAc and half sat. aq. NaHCO₃. The aqueous layer was extracted twice more with EtOAc and the combined organic layers were washed with brine, dried over MgSO₄ and concentrated. The crude product was purified on silica column, using a gradient of 0 to 25% EtOAc in heptane, to obtain 4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxy-benzaldehyde (71 mg).

iv) Analogous to the procedure described in **Example 1, step ii)**, the compound obtained in the previous step was converted into 4-{4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one (28 mg). MS(ES⁺) *m/z* 454.4 (M+H)⁺

78: 4-{4-[2-(difluoromethyl)-4-(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-

pyrazolo[3,4-b]pyridin-6-one



i) 4-Hydroxy-3-methoxybenzonitrile (250 mg), 2-fluoro-5-(trifluoromethyl)benzaldehyde (322 mg) and K₂CO₃ (463 mg) were suspended in DMF (8mL) and stirred at 90°C for 2,5 hours. The reaction was cooled to room temperature and partitioned between water and EtOAc. The water layer was extracted twice more with EtOAc and the combined organic layers were washed with brine, dried over MgSO₄ and concentrated. The crude product was purified on silica column, using a gradient of 0 to 30% EtOAc in heptane, to obtain 4-[2-formyl-4-(trifluoromethyl)phenoxy]-3-methoxy-benzonitrile (402 mg).

ii) Analogous to the procedures described in **Example 77, step ii)** to **step iv)**, the compound obtained in the previous step was converted to 4-{4-[2-(difluoromethyl)-4-(trifluoromethyl)phenoxy]-3-methoxyphenyl}-2H,4H,5H,6H,7H-pyrazolo[3,4-b]pyridin-6-one (78 mg). MS(ES⁺) *m/z* 454.4 (M+H)⁺

The single enantiomers of **Example 43** can be obtained by chiral separation. 2 g of racemic **Example 43** was dissolved in EtOH (50 mg/mL). The solution was injected numerous times on the chiral SFC using a preparative IG column (available from Daicel, amylose tris(3-chloro-5-methylphenylcarbamate) chiral stationary phase) and an isocratic gradient of 30% EtOH and 70% liquid CO₂, to obtain: 0.9 g of the (+)enantiomer (**Example 79**) at an Enantiomeric

Excess of 99.6%; and, 0.9 g of the (-)enantiomer (**Example 80**) at an Enantiomeric Excess 99.0%. The optical purity of the enantiomers was determined by chiral HPLC.

The absolute configuration of the compounds of Examples 79 to 86 is not known. These compounds were dissolved in DMSO (10mg/mL) and characterized by their optical rotation, using a Jasco P-2000 Polarimeter having the following configuration: Tungsten-Halogen (WI) light source; Glan-Taylor Prism Polarizer; Quartz Faraday Cell; and, a monitor wavelength of 589 nm.

79: (+)-4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

80: (-)-4-[4-[3,5-bis(trifluoromethyl)phenoxy]-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

Following a procedure analogous to that described for **Examples 79** and **80**, using appropriate starting materials and HPLC method, the following compounds have been prepared.

81: (+)-4-[4-(3,5-dimethylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

82: (-)-4-[4-(3,5-dimethylphenoxy)-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

83: (+)-4-[3-methoxy-4-[3-methyl-5-(trifluoromethyl)phenoxy]phenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

84: (-)-4-[3-methoxy-4-[3-methyl-5-(trifluoromethyl)phenoxy]phenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

85: (+)-4-[4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

86: (-)-4-[4-[3-(difluoromethyl)-5-(trifluoromethyl)phenoxy]-3-methoxyphenyl]-2*H*,4*H*,5*H*,6*H*,7*H*-pyrazolo[3,4-*b*]pyridin-6-one

Example 87

ERRα AlphaScreen Assay

This assay was based on the knowledge that nuclear receptors interact with cofactors in a ligand dependent matter.

The sites of interaction have been mapped to LXXLL motifs that are present in the co-activator sequences. Short peptide sequences that contain the LXXLL motif mimic the behavior of full length co-activators.

The ERRα AlphaScreen Assay described here relies on the interaction of the co-activator peptide with purified bacterial-expressed ERRα ligand binding domain (ERRα-LBD); upon ligand binding, the ERRα protein can undergo a conformational change resulting in a loss of co-activator binding.

Compounds of the present invention were tested for their ability to disrupt binding of ERR α -LBD protein to co-activator peptide using AlphaScreen® Technology (Perkin Elmer). ERR α -LBD protein was expressed in *E. coli* as a 6xHis Small Ubiquitin-like Modifier (SUMO) fusion. Bacterial expressed 6His-SUMO-ERR α -LBD protein was purified using affinity chromatography. All experiments were performed at room temperature in 384-well white non-binding plates (Greiner) using 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Pluronic F-127, 0.05% BSA and 5 mM TCEP as the buffer. Final DMSO concentration was 1% in the assay. Compounds were assayed in triplicate and incubated with 0.81 nM ERR α -LBD protein and 10 μ g/mL streptavidin donor beads and 10 μ g/mL Ni-chelate acceptor beads for 1 hour at room temperature; followed by a 2-hour incubation with 15 nM biotin-PGC1 α -3 peptide (QRRPCSELLKYLTNDPP) corresponding to amino acids 202 to 220.

The AlphaScreen signal was measured using an Envision Xcite plate reader (Perkin Elmer). Data was normalized, and curve fitting analysis was performed in GraphPad Prism 7 using a four-parameter dose-response fit.

All exemplified compounds of Formula I (Examples 1 – 86) were found to have mean pIC₅₀ values above or equal to 5.

Examples 1, 2, 4, 5, 6, 7, 14, 17, 20, 21, 22, 24, 25, 26, 49, 59, 60, 65, 66, 71, 72, 79, 81, 83 and 85 were found to have mean pIC₅₀ values above or equal to 6 but below 7.

Examples 10, 12, 15, 23, 28, 30, 31, 40, 41, 42, 43, 44, 45, 46, 47, 48, 52, 53, 54, 55, 56, 57, 58, 61, 64, 76, 77, 78 and 82 were found to have mean pIC₅₀ values above or equal to 7 but below 8.

Examples 27, 29, 32, 33, 34, 35, 36, 37, 38, 39, 63, 67, 68, 69, 70, 80, 84 and 86 were found to have mean pIC₅₀ values above or equal to 8.

Example 88

Full length ERR α reporter gene assay

Examples inhibitors 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85 and 86 were tested for their ability to inhibit ERR α activity in a full length ERR α reporter gene assay.

A method was established to quantitatively screen the potency of compounds with inverse agonistic activity on the nuclear receptor ERR α of the human species. The assay allows intra-cellular screening of ERR α inverse agonists in SK-BR-3 cells using an over-expression construct coding full length ERR α and a reporter construct containing an ERR α

Response Element (RE) and a luciferase gene for read out. The activity is expressed in logIC50 values and can be used to determine SAR of compound families or to de-select compounds.

In this assay, reporter cells are obtained by transient co-transfection of two constructs in SK-BR-3 cells using standard transfection techniques. The first construct contains a response element of the nuclear receptor ERR α (Plasmid pLP2175, Reporter construct ERR α -RE/luc2P, cloned variant of ERR α _v2_synthRE, Switchgear Genomics, Catalog Number S900089). This sequence drives the transcription of the luciferase reporter gene in response to binding of an ERR α protein encoded by the second construct (Plasmid pLP2124: full length ERR α expression construct using pcDNA3.1/Hygro(+) as background, Invitrogen Catalog Number V87020). The over-expressed full length ERR α is constitutively active, hence luciferase expression is reduced by inverse agonists of the nuclear receptor ERR α .

- 10 The day after transfection, cells were plated into 96 well plates, the test compound was added and the plates were incubated overnight. Subsequently, the firefly luciferase activity was quantified using luciferase detection reagent and luminescence readout.

Detailed assay description

- 15 Transfection is performed on pre-seeded SK-BR-3 cells in a T175 flask. One transfected T175 flask is sufficient for seeding 3 to 4 MW96 plates the next day, depending on the confluency of the transfected cells.

Two different media are used in this protocol for cell treatment: 1) Culture medium, i.e. McCoy's 5a with phenol red (BioWhittaker Supplier Number 12-688F), 10% FBS and 1x Penstrep.; and, 2) Assay medium, i.e. McCoy's 5a Medium phenol red free (HyClone Product Code SH30270.01) with 2% Charcoal Stripped FBS and 1x Penstrep. Compound dilutions are prepared in assay medium.

- 20 Cells are seeded at least 2 days in advance to allow the cells to adhere well to flask before transfection. Cells should be 50-80% confluent at the day of transfection.

SKBR3 cells were transfected with the transcriptional reporter construct pLP2175 and the ERR α expression construct pLP2124 (as described above).

- 25 68 μ L of Lipofectamine LTX transfection reagent (Invitrogen Catalog Number 15338-100) was added dropwise to 8.9 ml Opti-MEM I Reduced Serum Medium (Gibco Catalog Number 51985-026) and incubated at room temperature for 5 to 20 minutes. 8.9 ml of this reagent mixture was added to 22 μ g pLP2175 + 22 μ g pLP2124 (ratio 1:1 and total volume 10 ml), and incubated at room temperature for 25 minutes.

- 10 minutes before adding the transfection mix to SKBR3 cells in a T175 flask, the culture medium was refreshed with 20 mL culture medium. Subsequently, the 10 mL DNA-Opti-MEM-Lipofectamine mixture was gently added to the cells, 30 followed by overnight (16-24 hours) incubation at 37°C and 5% CO₂.

To harvest SKBR3 cells from a T175 flask, first the medium was removed. Subsequently, cells were washed with Phosphate Buffered Saline (PBS) (Lonza), after which the PBS was removed. To dissociate the cells, 2 ml of TrypLE Express (Invitrogen) was added to the flask, followed by incubation at 37°C for 5 minutes. The flask was tapped until cells were detached from the bottom. Cells were collected in 8 mL of culture medium (McCoy's 5a, 10% FBS, penstrep),
5 to achieve a single cell suspension. After cell count, cells were spun down for 5 minutes at 300 g. Next cell pellets were re-suspended to 25000 cells/80 µl assay medium (McCoy's 5a phenol red free, 2% charcoal stripped FBS, penstrep).

For compound screening, the cells were harvested (as described above). 80 µL of cell suspension (25,000 cells) was plated per well into a white, flat bottom, tissue culture treated, 96 well screening plates (Greiner).

Test compounds were diluted, starting from a 10 mM dimethylsulfoxide (DMSO) stock solution, in 3 dilution steps. The
10 first dilution step was a 12 points serial dilution of 4-fold in DMSO. These dilutions were further diluted 10 times in phenol red free assay medium containing 2% charcoal stripped FBS and penstrep. The last step was another 20-fold dilution in assay medium to obtain a 5x concentrated dilution with a DMSO concentration of 0.5%. As a last step, the compound dilutions were diluted 5x in the cell plate.

The DMSO dilution series consisted of 12 concentrations, with a final concentration in the cell plate ranging from 10 µM
15 to 2.4 fM.

The plates were incubated overnight (16-24 hours) at 37°C and 5% CO₂.

For the luciferase readout, the luciferase reagent (BriteLite Plus, Perkin Elmer) was brought to room temperature. To each test well of the screening plates, 100 µL of 2.5-fold diluted BriteLite Plus reagent was added, followed by incubation at room temperature for 5 minutes. The luciferase luminescence signal was measured using a Wallac Victor Microplate
20 Reader (Perkin Elmer).

The half maximum inhibitory concentration (IC₅₀) values for the test compounds were calculated from the luciferase signal using GraphPad Prism software (GraphPad Software).

From the exemplified compounds of Formula I, Examples **10, 29, 32, 33, 34, 35, 37, 38, 39, 40, 41, 43, 63, 67, 68, 69, 70, 84** and **86** were found to have mean pIC₅₀ values above or equal to 6.5.

25

Example 89

B16F10 Melanoma Syngeneic Mouse Model

Example inhibitor **80** was tested for its ability to inhibit tumor growth in a B16F10 melanoma syngeneic mouse model.

Cell line and tumor model: B16F10 melanoma cell line derived allograft model in C57BL/6 mice.

Mouse B16F10 melanoma cells were sourced from American Type Culture Collection (ATCC), USA. Cells were grown in DMEM (Invitrogen, Catalogue No. 31600-034) supplemented with 10% FBS (Invitrogen, Catalogue No. 10438-026), and 1% penicillin streptomycin (Thermo Fisher Scientific, Catalogue No. 15140-122). To establish allografts, the cells were harvested by trypsinization when they reach around 70 to 80 % confluence and 0.1 million B16F10 cells were suspended in 50 μ l of serum free medium and mixed at 1:1 ratio with matrigel before implanting subcutaneously into the dorsal right flank of mice using a 1 mL BD syringe attached to a 24-gauge needle.

B16F10 tumor grafts were measured after 5 days of cell inoculation once they became palpable. When the average tumor volume reached around 58 mm³, animals were dosed after randomization into different treatment groups keeping tumor volume and number of animals in such a way so that the average tumor volume of each group remained same across the groups. Dosing was performed on a per kilogram weight basis, by mouth (*per os*, *p.o.*) once a day (quaque die, *q.d.*).

Tumor dimensions - length (l) and breadth (b) - were measured by caliper on the day of animal randomization based on tumor volume (Day 1) and thrice weekly thereafter until study termination. Tumor volumes were calculated using the formula $b^2 \times l \times 0.52$ where l=length, b=breadth (Dusan Djokovic et al., BMC Cancer, 2010, 10:641). Tumor growth inhibition was calculated after normalizing the tumor volume on a given day to that on Day 1.

Test items administration was started when tumor volume reached an average of 58 mm³.

Administration of test items was carried out until Day 14 and measurement of tumor volume was carried out until Day 13 for computing percent tumor growth inhibition (TGI). The results of the study are listed in Table 1 herein below.

Table 1

Example	Dose Milligrams per kilogram weight (mpk) [p.o., q.d]	Tumor Volume (mm ³) Day 13	Tumor Growth Inhibition (%)
Vehicle Control		1801 $\pm$ 241	-
80	30	684 $\pm$ 82	64

In view of the foregoing description and examples, it will be apparent to those skilled in the art that equivalent modifications thereof can be made without departing from the scope of the claims.