Expeditious Synthesis and Biological Characterization of Enantio-enriched (-)-Nutlin-3

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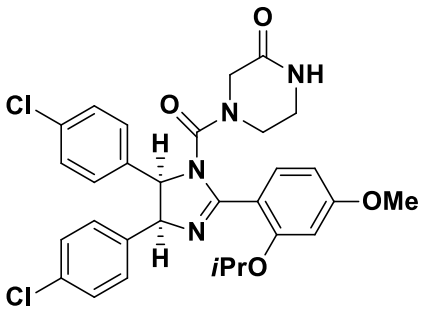
Abstract: A concise and operationally simple synthesis of enantio-enriched Nutlin-3 featuring the desymmetrization of 1,2-bis(4-chlorophenyl)ethane-1,2-diamine as the key step is described. The easy-to-make *N*-((1*R*,2*R*)-2-(3-(3,5-bis(trifluoromethyl)phenyl)thioureido)cyclohexyl)-3,5-bis(trifluoromethyl)benzamide (and *N*-((1*S*,2*S*)-2-(3-(3,5-bis(trifluoromethyl)phenyl)thioureido)cyclohexyl)-3,5-bis(trifluoromethyl)benzamide)) involved as anion binding in the key desymmetrization step is the completely recoverable chiral auxiliary. An array of biological tests performed with the 84:16 **(-)-Nutlin-3** scalemic mixture showed activities comparable to those of the commercial eutomer.

Introduction

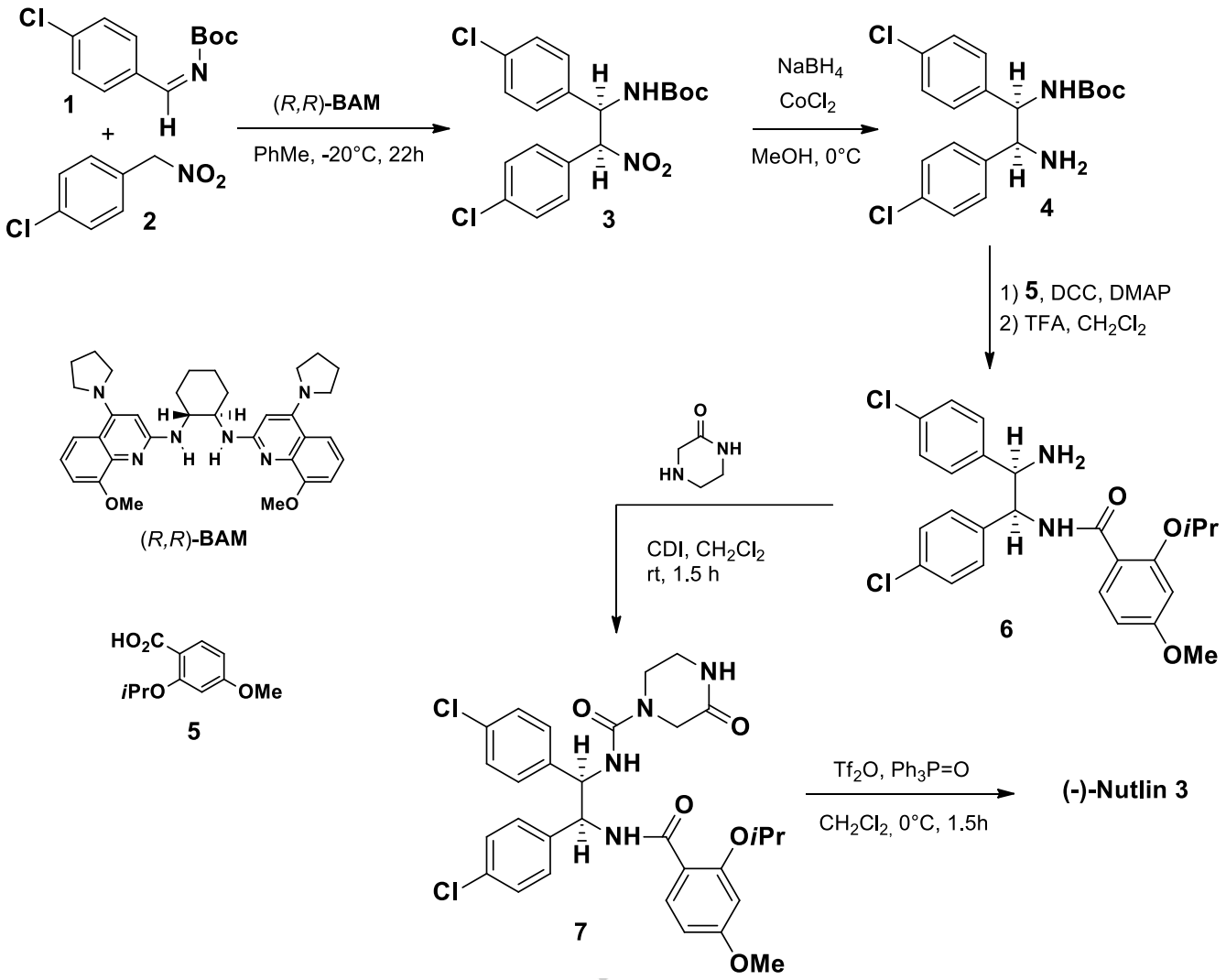
In 2004, scientists from Hoffmann-La Roche discovered the first class of highly potent, specific, and orally active MDM2 inhibitors featuring a tetrasubstituted imidazoline scaffold, known as Nutlins.[1,2] Modifications and optimizations resulted in a host of derivatives, the most potent in this series being **(-)-Nutlin-3** which revealed a promising *in vitro* and *in vivo* antitumor activity.[3]



(-)-Nutlin 3

Both experimental and computational studies have shown that Phe^{19}, Trp^{23}, and Leu^{26} residues in p53 α-helix are the crucial residues for the interaction with the binding cleft of MDM2, while Nutlin-3 bound to MDM2 shows that both 4-chlorophenyl groups perfectly fill the Leu^{26} and Trp^{23} pockets and the isopropoxy group reaches deep into the Phe^{19} pocket.[4] Notably, Hoffmann-La Roche entered one of the member of this family into phase I clinical trials against advanced solid and soft-tissue tumors and hematological

malignancies.[5,6] The intriguing overcrowded structure of nutlins stimulated intensive chemical research aimed at developing efficient and flexible synthetic strategies for their preparation.



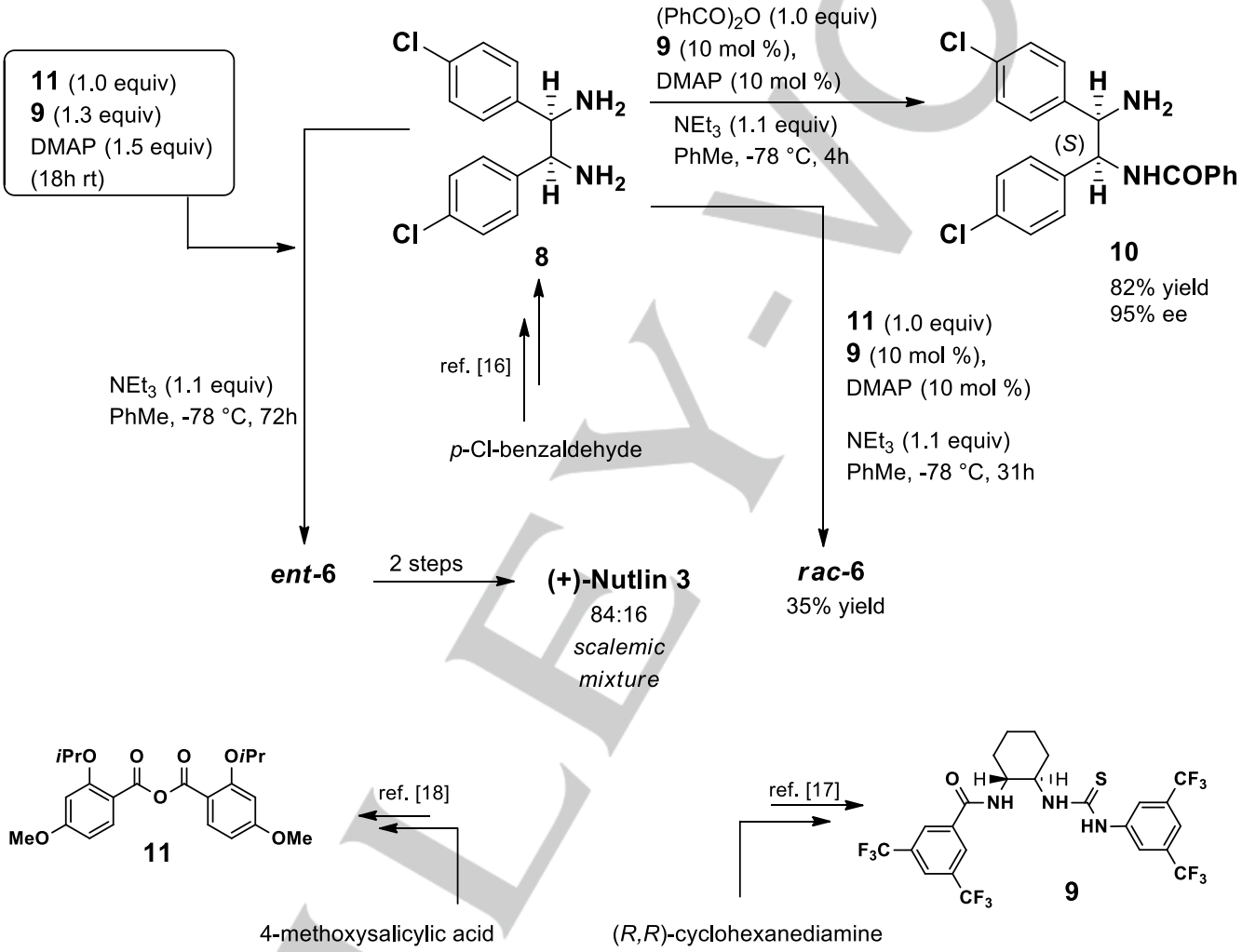
Scheme 1. Johnston and co-workers route to (-)-Nutlin 3

Thus, Nutlin-3 in racemic form could be obtained following the Hoffmann-La Roche synthesis reported both in patents[7] as well as in two papers[8] in late 2012 but the obtainment of the more potent enantiomer required the need for supercritical fluid chromatography.[9]

A series of paper by Johnston and co-workers[10-12] described the first catalytic enantioselective synthesis of (−)-Nutlin-3 featuring a crucial carbon-carbon bond-forming addition of aryl nitromethanes to imines, producing aza-Henry adducts at noncryogenic temperature (−20 °C) in high levels of stereocontrol thanks to the identification of an electron rich chiral Bis(Amidine) catalyst (BAM) and later to the discovery of novel mono(amidine) organocatalysts (MAM). Thus, the aza-Henry adduct **3** obtained by BAM-catalyzed reaction of the *N*-Boc imine **1** and the aryl nitroalkane **2** underwent nitro group reduction with $CoCl_2/NaBH_4$ to produce the free amine **4**. The latter compound was first acylated with 2-isopropoxy-4-methoxybenzoic acid **5** using EDC coupling conditions, later submitted to standard TFA-promoted Boc removal. Thus, the monoamide **6**, obtained as a white solid, could be taken to (-)-Nutlin-3 by further two steps including carbamoylation reaction with 2-oxopiperazine to give **7** and final Hendrickson's dehydrative cyclization.[13] Notably, the procedure

has been used to prepare 17 g of (−)-Nutlin-3 in a single batch, with column chromatography necessary after only three of the steps.[12] This very nice synthetic approach is depicted in the Scheme 1.

However, the relevant number of steps, the starting materials themselves not being easily to make compounds, our own interest in the field[14] coupled with the significant financial hurdle represented by the price of (−)-Nutlin-3 from commercial sources prompted us to examine the possibility to open a shorter synthetic route shown in Scheme 2.



Scheme 2.

Results and Discussion

We were attracted to apply the Seidel's desymmetrization approach[15] of *meso*-1,2-diaryl-1,2-diaminoethanes in the presence of benzoic anhydride, DMAP as a nucleophilic catalyst, and the thiourea-amide **9** as a benzoate-receptor co-catalyst.

The ternary organocatalyst complex resulting from the ion pairing of the achiral benzoyl pyridinium cation and the chiral thiourea-amide benzoate allowed for the preferential benzoylation

of the (*S*) configured benzylamino group in a small library of *meso*-1,2-diaryl-1,2-diaminoethanes including **8**. The latter being readily available by reaction of *p*-Cl-benzaldehyde with ammonium acetate following reported directions.[16]

We were interested to apply this protocol using 2-isopropoxy-4-methoxybenzoic acid anhydride **11**, instead of benzoic anhydride, as acylating agent of the *meso*-diamine **8** thus shortening the approach to the monoamide **6** already described by Johnston and co-workers[12] avoiding the required tedious protection-deprotection steps.

To this end, the chiral hydrogen-bonding anion receptor **9** required for the selective acylation of the (*S*)-configured amino group of **8** according to Seidel's findings[17] was prepared starting from 1(*R*),2(*R*)-cyclohexanediamine while the *meso*-diamine **8** was conveniently obtained by reaction of *p*-Cl-benzaldehyde with ammonium acetate entailing on a stereospecific disrotatory ring closure.[16] The requisite anhydride **11** was synthesized from commercially available 4-methoxysalicylic acid through dialkylation with isopropyl bromide in the presence of potassium carbonate followed by saponification of the dialkylated compound.[18] Eventually, the free acid underwent smoothly $TsCl/K_2CO_3$-promoted intermolecular dehydration to the corresponding anhydride **11**.

Having in hands the required reagents, we accomplished the monobenzoylation of **8** following the directions established by De and Seidel. Actually, the monoamide **10** could be obtained in yield and optical purity overlapping data in the literature.[15]

Instead, and not completely unexpectedly, the related reaction with anhydride **11** gave rise to the anticipated monoamide essentially as a racemate. We presume that, due to steric reason, the assembly of the ternary organocatalyst complex is prevented thus ***rac*-6** was produced under non catalytic conditions. In fact, recent studies by Seidel and co-workers[19] on kinetic resolution of racemic benzylic amines *via* organo-catalyzed amidation showed that substituted benzoic anhydrides, without exception, provided poorer results without showing a discernible trend with regard to electronics.

Therefore, we were forced to evaluate different reaction parameters to improve the pivotal mono-amidation reaction with gradual increasing of the thiourea catalyst loading and/or reaction time (Table 1 Supporting Information). We were aware that the monoacylation of a diamine is challenging since the resulting monoamide is usually more reactive than the diamine.[20] Actually, the a posteriori stereo-defined amino amide ***ent*-6** was recovered in 47% yield and 68% ee submitting a cooled (-78 °C) toluene solution of *meso*-diamine **8** for 72h to the action of pre-formed chiral acyl-transfer reagent. The latter, was in turn prepared mixing the anhydride **11**(1.0 equiv.), DMAP (1.5 equiv.), and the amide-thiourea **9** (1.3 equiv.) for 18h at rt.

Much to our surprise, the positive optical power measured for the Nutlin-3 produced following Johnston directions[12] revealed that the undesired antipode was the predominant enantiomer in the scalemic mixture.

For reasons that are not yet clear, in our hands the desymmetrization reaction of **8** took place with reversal of the enantioselectivity as compared to the closely related Seidel's organo-catalyzed mono benzoylation.[15] We cannot exclude a different nature of the aroyl-transfer reagent involved in the acylation step in comparison to the one advanced by Seidel and co-workers. Thus, in order to produce the (-)-Nutlin-3 eutomer, we were forced to prepare ***ent*-9** starting from 1(*S*),2(*S*)-cyclohexanediamine. As expected, we were able to obtain levorotatory Nutlin-3 as 84:16 scalemic mixture. Noteworthy, the chiral auxiliary thiourea ***ent*-9** could be recovered almost-quantitatively from the reaction mixture by column chromatography.

Biological results

In order to functionally validate scalemic-Nutlin, we have performed a combination of biological assays aimed at assessing its ability to activate p53 pathway. For this purpose scalemic-Nutlin was comparatively tested on $p53^{wild\text{-}type}$ (EHEB and JVM-2) as well as on $p53^{mutated/deleted}$ (BJAB and HL-60) leukemic cell lines used as control of specificity. In parallel, cell cultures were exposed to commercial Nutlin-3 used as positive control and for comparison. As shown in Figure 4, treatment with Nutlin-3 and scalemic-Nutlin, used in the range of 1-10 μM for up to 48 hours, exhibited a comparable dose-dependent cytotoxicity resulting in a significant reduction of cell viability (Figure 4A) coupled to apoptosis induction (Figure 4B) and cell cycle arrest (Figure 4C) specifically in $p53^{wild\text{-}type}$ but not in $p53^{mutated/deleted}$ cell lines. Consistently with the observed biological effects, molecular analysis of p53 pathway by protein (Western Blot) and RNA (quantitative RT-PCR) documented accumulation of p53 and transcriptional induction of two p53 transcriptional canonical targets, involved in promoting cell cycle arrest (p21) and modulation of apoptosis (MDM2), in response to treatment with scalemic-Nutlin (Figure 4D).

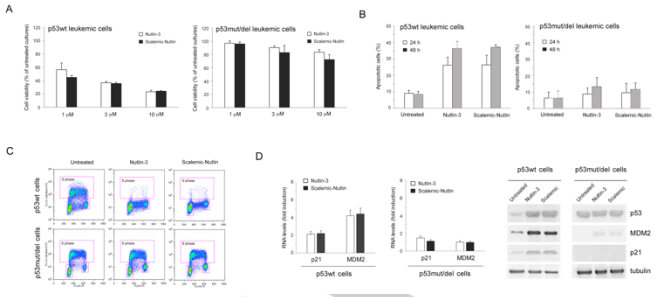


Figure 4 Effects of Nutlin-3 and scalemic-Nutlin on leukemic cell lines. $p53^{wild\text{-}type}$ as well as $p53^{mutated/deleted}$ B leukemic cell lines were exposed to Nutlin-3 or scalemic-Nutlin (range 1-10 μM) for the times indicated in each set of experiments. In A, cell viability in response to serial doses of both Nutlin-3 and scalemic-Nutlin (range 1-10 μM), was calculated at 48 hours of treatment as percentage with respect to the control vehicle cultures (set to 100%). In B, the percentage of apoptotic cells in response to Nutlin-3 or scalemic-Nutlin (each used at 10 μM) was determined by flow-cytometry after annexin-V/PI staining. In A and B, data are reported as the mean±SD of results from four independent experiments. The asterisk indicates $p<0.05$ with respect to the untreated cultures of each cell line. In C, cell distribution in the different phases of the cell cycle was analyzed by flow cytometry after BrdU/PI staining. Representative cell cycle profiles of cultures, either left untreated or treated with Nutlin-3 or scalemic-Nutlin, analyzed by flow cytometry are shown. For each cytofluorimetric analysis, the rectangles represent the cells in G0/G1, S, G2/M phases of the cell cycle. In D, the expression levels of p53 target genes, p21 and MDM2, were assessed by quantitative RT-PCR and results were indicated as folds of modulation with respect to the control untreated cultures set at 1 (left panels). Data are reported as mean±SD of results from independent experiments. The asterisk indicates $p<0.05$ with respect to untreated cultures. In the right panels, representative Western Blot results documenting modulation of MDM2, p53 and p21 proteins by Nutlin-3 or scalemic-Nutlin are shown.

Moreover, as shown in Figure 4, the effects induced by scalemic-Nutlin was equally effective as commercial Nutlin-3.

Conclusions

An expeditious synthetic approach to enantio-enriched (-)-Nutlin-3 has been developed by exploiting an unprecedented chiral thiourea-promoted enantioselective *N*-acylation of *meso*-1,2-diaryl-1,2-diaminoethane **8**. Noteworthy, the enantioselectivity we observed in the key desymmetrization step was opposite to the one found by Seidel *et al*. in their innovative related catalytic approach. Our protocol is advantageous in terms of time-saving, atom-economy and costs. The need of stoichiometric amounts of the chiral thiourea ***ent*-9** was partially compensated by its easy recover through chromatographic purification. Our biological tests clearly established that the scalemic (-)-Nutlin-3 we reached was as effective as the commercial eutomer in activating the p53 pathway.

Experimental Section

Chemistry

General

The reactions and the mixtures of products were checked by thin layer chromatography (TLC) on plates of silica gel Macherey-Nagel SIL Poligram / UV 254 by 0.25 mm, using a

UV lamp at 254 nm and potassium permanganate in aqueous solution at 2% as detection systems. The products were dried with anhydrous sodium sulfate (Na_2SO_4) from Carlo Erba. The optical rotation was recorded with the instrument Perkin Elmer 241polarimeter.

The ^{1}H-NMR, ^{13}C-NMR, DEPT, two-dimensional (COSY) and eterocorrelated (HMBC, HMQC) spectra were carried out with a Varian 400 MHz instrument in $CDCl_3$ solution unless specified differently. The chemical shifts (δ) are expressed in part

per million (ppm), using the solvent peak as internal standard, while the coupling constants (*J*) are reported in Hertz (Hz). For multiplicity apply the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; bs = broadened signal; dd = double doublet; dt = double triplet; td = triple doublet.

The molecular weights of the intermediates and final products were determined by a mass spectrometer, electrospray ESI MICROMASS ZMD 2000. The exact masses were determined with a HPLC-ESI-Q-TOF instrument 6520 (Agilent Technologies) with Chip Cube® system. The products were purified with a medium pressure chromatographic system Isolera 1 (Biotage, Sweden). The chiral HPLC spectra were obtained with an Agilent Infinity 1200 (Agilent Technologies) and the conditions are reported for individual chromatograms.

1,2-bis(4-chlorophenyl)ethane-1,2-diamine (8) In a 100 mL flask, equipped with magnetic stirrer and bubble condenser, 4.8 g (8.85 mmol) of benzylbenzoyl amide and 80 mL of 50% (v/v) solution of H_2SO_4 were placed. The mixture was heated at 170 ° C and was stirred for 24 h. After having cooled to room temperature, the mixture was diluted with 30 mL of water, transferred to a 500 mL separatory funnel and washed twice with 20 mL Et_2O. The aqueous phase was basified to pH 9 by slowly adding a solution of NaOH 2N, and was extracted with Et_2O in a separatory funnel. The organic phase was dried over Na_2SO_4, filtered over cotton and 1.98 g (7.03 mmol; yield 79%) of the pale yellow solid of *meso*-diamine **8** was obtained after removing the solvent by evaporation. Mp 128-130 ° C; TLC: R_f (ethyl acetate / petroleum ether 1: 3) = 0,18; Calc $[M+H]^+$ = 281.06068, $[M-NH_2]^+$ 264.0341; Mis $[M+H]^+$ = 281.06076, $[M-NH_2]^+$ 264.03422; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.37 - 7.18 (m, 8H), 3.99 (s, 2H), 1.52 (s, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.07, 133.53, 129.05, 128.72, 128.40, 62.1.

N-((1S,2R)-2-amino-1,2-bis(4-chlorophenyl)ethyl)-2-isopropoxy-4-methoxybenzamide (*ent*-6) In a 250 mL two-neck round bottom flask equipped with magnetic stirrer and rubber septum in pure Ar atmosphere were placed 200 mg of 4Å MS and a solution of anhydride **11** (300 mg, 0.745 mmol) in freshly distilled toluene (10 mL, 0.01 M). Then 137 mg (1.12 mmol, 1.5 equivalents) of DMAP, dissolved in 5 mL of anhydrous toluene were added to the flask. The mixture was stirred at room temperature for 18 h, then was cooled to -78°C. In a 10 mL Erlenmeyer flask were dissolved 600 mg (0.96 mmol, 1.3 equivalents) of **9** in 20 mL of anhydrous toluene and 10 mL of ethyl acetate, and the solution was added in the reaction flask using a syringe. After stirring for 30 min, 210 mg (0.746 mmol, 1 equivalent) of meso-diammine **8**, dissolved in 40 mL of anhydrous toluene, were added trhough a dropping funnel for 1 h. Later 114 µL (0.728 mmol, 1.1 equivalents) of freshly distilled Et_3N were added. The reaction was quenched after 72h with 10 mL of a methanolic ammonia solution and washed with a 1: 1 mixture of water and brine. The organic phase was dried over Na_2SO_4, filtered over cotton and and the solvent was removed by evaporation with rotatory evaporator, to obtain a gummy yellow solid. The crude product was purified by gradient elution chromatography on silica gel (25-100% petroleum ether / EtOAc), to obtain 166 mg (0.35 mmol) of amide ***ent*-6**, a white solid. (Yield 47%, enantiomeric excess *ee%* 68%). Mp = 181-185°C; TLC: Ethyl acetate/ petroleum ether (7 : 3) R_f = 0,43; TLC: MeOH/ CH_2Cl_2 (0.5 : 9.5) R_f = 0,67; HPLC-FSC (Chiralpack-ID 250mm x 4.6 mm, 5µm. MP: 50% 2-propanol 50% esane. 1mL/min. Loop: 5µL; 254 nm): t_R [***ent*-6**]=22.93 min, 80 %, t_R [**6**]=39.77min, 20 % ($ee_{\%}$ = 68%). $[\alpha]_d$ = -9.3 (0,1 *c*, MeOH); Calc $[M+H]^+$ = 473,13930; Mis. $[M+H]^+$ = 473,13932; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.84 (d, *J* = 7.8 Hz, 1H), 8.11 (d, *J* = 8.8 Hz, 1H), 7.29 – 7.16 (m, 5H), 7.01 (dd, *J* = 8.5, 3.0 Hz, 4H), 6.55 (dd, *J* = 8.8, 2.3 Hz, 1H), 6.48 (d, *J* = 2.3 Hz, 1H), 5.47 (dd, *J* = 7.9, 4.6 Hz, 1H), 4.74 (p, *J* = 6.1 Hz, 1H), 4.43 (d, *J* = 4.6 Hz, 1H), 3.83 (d, *J* = 0.7 Hz, 3H), 1.41 (t, *J* = 5.8 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.08, 163.51, 157.37, 140.00, 136.84, 134.31, 133.42, 129.26, 128.56, 128.41, 114.95, 105.30, 100.51, 71.67, 59.25, 58.63, 55.66, 22.33, 22.18.

N-((1S,2R)-2-amino-1,2-bis(4-chlorophenyl)ethyl)-2-isopropoxy-4-methoxybenzamide (6) Same conditions for **(*ent*-6)** but using ***ent*-9** as a chiral auxiliary: HPLC-FSC (Chiralpack-ID 250mm x 4.6 mm, 5µm. MP: 50% 2-propanol 50% esane. 1mL/min. Loop: 5µL; 254 nm): t_R [***ent*-6**]=22.93 min, 16.4 %, t_R [**6**]=38.47 min, 83.6 % ($ee_{\%}$=68%); $[\alpha]_d$ = + 7.0 (0,1 *c*, MeOH).

N-((1R,2S)-1,2-bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxybenzamido)ethyl)-3-oxopiperazine-1-carboxamide (*ent*-7) In a 100 mL two-neck round bottom flask, previously flame-dried, equipped with a magnetic stirrer and rubber septum, in Ar atmosphere, 293 mg (0.62 mmol) of amine ***ent*-6** were dissolved in 25 mL of anhydrous CH_2Cl_2 and 120 mg (0.742 mmol, 1.2 equivalents) of CDI were added. The mixture was stirred at room temperature for 5 h and then 99.15 mg (0.99 mmol, 1.6 equivalents) of 2-piperazinone were added. After 18 h, the mixture was diluted with 30 mL of anhydrous CH_2Cl_2, was washed three times with water (60 mL) and once with brine (40 mL). The organic phase was dried over Na_2SO_4, filtered over cotton and the solvent was removed by evaporation with rotatory evaporator, to obtain a 350 mg (0.58 mmol) of urea ***ent*-7**, as a white solid (94% yield). Mp = 210-213 °C; TLC MeOH/ CH_2Cl_2 (0.5 : 9.5) R_f = 0,40; $[\alpha]_d$ = - 28.5 (0,1 *c*, $CHCl_3$); Calc $[M+H]^+$ = 599.182252; Mis $[M+H]^+$ = 599.18294; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.38 (d, *J* = 7.7 Hz, 1H), 8.26 (d, *J* = 8.8 Hz, 1H), 8.02 (s, 1H), 7.82 (s, 1H), 7.28 (d, *J* = 8.2 Hz, 2H), 7.17 (d, *J* = 8.2 Hz, 2H), 6.95 (d, *J* = 8.2 Hz, 2H), 6.89 (d, *J* = 8.1 Hz, 2H), 6.62 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.45 (d, *J* = 2.1 Hz, 1H), 5.77 (d, *J* = 7.6 Hz, 1H), 5.09 (s, 1H), 4.65 (p, *J* = 6.1 Hz, 1H), 4.15 (s, 2H), 3.85 (s, 3H), 3.72 (d, *J* = 13.0 Hz, 1H), 3.60 (d, *J* = 12.6 Hz, 1H), 3.41 (s, 2H), 1.20 (d, *J* = 6.0 Hz, 3H), 1.14 (d, *J* = 6.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.48, 164.17, 157.44, 156.09, 136.56, 134.64, 134.08, 133.51, 129.60, 128.83, 128.58, 128.22, 113.56, 105.55, 100.50, 71.64, 62.11, 57.94, 55.77, 47.61, 41.40, 40.12, 22.12, 21.73.

N-((1S,2R)-1,2-bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxybenzamido)ethyl)-3-oxopiperazine-1-carboxamide (7) Same procedure for the synthesis of ***ent*-7**. $[\alpha]_d$ = + 29.7 (0,1 *c*, $CHCl_3$).

2-isopropoxy-4-methoxybenzoic acid (5) A 100 mL flask, equipped with magnetic stirrer and bubble condenser was charged with a solution of 4.17 g (16.5 mmol) of the

correspondig isopropylester in 30 mL of EtOH and 33 mL of a 2N solution of NaOH (66.1 mmol, 4 equivalents) were added. The mixture was stirred at reflux temperature for 2.5 h, then was cooled to room temperature and acidified with a 2N HCl solution untill pH 3. The mixture was poured into a separatory funnel and extracted with EtOAc (2X50mL); the organic phases were dried over Na_2SO_4, filtered over cotton and the solvent was removed by evaporation with rotatory evaporator, to obtain 3.26 g (15.5 mmol; yield 94%) of acid **5**, as a viscous liquid. ^{1}H NMR (200 MHz, Chloroform-*d*) δ: 10.83 (bs, 1H), 8.14 (d, J= 9 Hz, 1H), 6.64 (dd, J= 8.8, 2.4 Hz, 1H), 6.52 (d, J= 2.4 Hz, 1H), 4.80 (p, J= 6 Hz, 1H), 3.85 (s, 3H), 1.48 (d, J= 6.2 Hz, 6H). IR (neat): 3262.83, 2979.76, 1726.91, 1564.43 cm $^{-1}$.

2-isopropoxy-4-methoxybenzoic anhydride (11) 235 mg (1.12 mmol) of acid **5** and 670 mg (4.85 mmol, equivalent 4.34) of K_2CO_3 were mixed in a mortar with a pestle, then a solution of tosyl chloride (128 mg, 0.67 mmol, 0.6 equivalent) in AcOEt (4 mL) was added dropwise. The preparation was pounded and mixed for 20 min by adding a few drops of EtOAc to make it more homogeneous. Subsequently 15 mL of CH_2Cl_2 were added, the mixture was filtered on a Gooch funnel and the supernatant was dried with rotary evaporator, to obtain 218 mg of anhydride **11** (0.54 mol; yield 96%), as a white solid. ^{1}H NMR (200 MHz, Chloroform-*d*) δ: 8.02 (d, J= 9 Hz, 2H), 6.47 (m, 4H), 4.58 (p, J= 6 Hz, 2H), 3.85 (s, 6H), 1.33-1.20 (m, 12H). IR: 2980.15, 2843.06, 1737.45, 1715.92, 1603.27, 1566,35 cm^{-1}.

4-((4S,5R)-4,5-bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxyphenyl)-4,5-dihydro-1H-imidazole-1-carbonyl)piperazin-2-one, (-)-Nutlin-3. In a 100 mL two-neck flask, previously dried, equipped with a magnetic stirrer and rubber septum, 328 mg (1.175 mmol, 4 equivalents) of triphenyl phosphine oxide were dissolved in 25 mL of anhydrous CH_2Cl_2 at 0 ° C, in Ar atmosphere. After 10 min, 198 µL (1.175 mmol, 4 equivalents) of triflic anhydride were added to the solution and the mixture was stirred for 30 min, then a solution of compound **7**, (176 mg; 0.294 mmol) in anhydrous CH_2Cl_2 (15 mL) was added, at 0 ° C. The mixture was stirred for 18 h from 0°C up to room temperature. When the disappearance of the starting reagent **7** was detected by TLC, the reaction was stopped by slowly adding a saturated solution of $NaHCO_3$ (30 mL). The two phases were transferred to a separator funnel and the organic phase was dried over Na_2SO_4, filtered over cotton and the solvent was removed by evaporation with rotary evaporator. The crude product was purified by gradient elution chromatography on silica gel (0-4% MeOH / CH_2Cl_2), to obtain 136 mg (0.234 mmol; yield 80%) of **(-)-Nutlin-3**, a white solid. Mp = 143-147°C; TLC MeOH/CH_2Cl_2 (0.5 : 9.5) R_f = 0,23; $[\alpha]_d$ = - 53,67 (0,1 *c*, $CHCl_3$); Calc $[M+H]^+$ = 581.1717 ; Mis $[M+H]^+$ = 581.1729; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 – 7.56 (m, 2H), 7.08 (d, *J* = 8.2 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 6.93 (d, *J* = 8.4 Hz, 2H), 6.87 (d, *J* = 8.1 Hz, 2H), 6.62 (s, 1H), 6.55 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.47 (d, *J* = 2.1 Hz, 1H), 5.66 – 5.56 (bs, 1H), 4.61 (p, *J* = 6.0 Hz, 1H), 3.84 (s, 3H), 3.77 (d, *J* = 18.2 Hz, 1H), 3.63 (d, *J* = 18.3 Hz, 1H), 3.40 – 3.37 (m, 1H), 3.27 – 3.15 (m, 1H), 3.00 (s, 2H), 1.38 (d, *J* = 6.0 Hz, 3H), 1.33 (d, *J* = 6.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.76, 163.63, 161.07, 157.30, 154.33, 135.46, 134.58, 133.55, 133.24, 132.63, 129.31, 128.63, 128.29, 128.18, 104.92, 100.30, 71.29, 69.41, 55.74, 49.51, 42.09, 40.53, 22.19.

Biology

Functional characterization of scalemic-Nutlin, obtained by our synthetic procedure, was performed by *in vitro* studies on $p53^{wild\text{-}type}$ (EHEB and JVM-2) and $p53^{mutated/deleted}$ (BJAB, HL-60) leukemic cell lines (all purchased from DSMZ, Braunschweig, Germany). In all experiments, the activity of the scalemic-Nutlin was compared to commercial Nutlin-3 (CaymanChemical, Ann Arbor, MI). For the assays, cells were seeded at a density of $1x10^6$ cells/ml and exposed to different doses (1–10µM) scalemic-Nutlin, for the times indicated in each set of experiments. Cells were then analyzed for cell viability, apoptosis and cell cycle. Cell viability was examined by Trypan blue dye exclusion and MTT (3-(4,5-dimethilthiazol-2yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Roche Diagnostics Corporation, Indianapolis, IN) for data confirmation, as previously described[21]. Levels of apoptosis were quantified by Annexin V-FITC/propidium iodide (PI) staining (Immunotech, Marseille, France) followed by analysis using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA)[22]. The cell cycle profile was analyzed by flow cytometry after 5-bromodeoxyuridine (BrdU) incorporation. Briefly, leukemic cells were incubated with 50 µM BrdU (Sigma-Aldrich, St. Louis MO) at 37°C for 1 hour. Antibody anti-BrdU (BD Biosciences Pharmingen, San Diego, CA) was bound to BrdU incorporated into neosynthesized DNA and the complex was detected by FITC conjugated secondary antibody (Immunotech). Cells were then stained with PI (50 µg/mL) and analyzed by flow cytometry[23]. The modulation of protein and RNA expression were evaluated with western blot and RNA analyses. For western blot, cell lysates were obtained as previously described[24], supplemented with loading buffer (250 mM Tris pH 6.8, 2% SDS, 10% Glycerin, 4% beta-mercaptoethanol, 1% bromophenol blue) and then boiled for 2 min. Equal amounts of protein for each sample were migrated in SDS-polyacrylamide gels and blotted onto nitrocellulose filters. The following antibodies were used: anti-p53 (DO-1), anti-MDM2 (SMP14) and anti-p21 (C-19) (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-tubulin (from Sigma-Aldrich). Total RNA was extracted from cells using the QIAGEN RNeasy Plus mini kit (QIAGEN, Hilden, Germany) according to the supplier's instructions. Total RNA was transcribed into cDNA and amplified using the Express One-Step Superscript qRT-PCR Kit (Invitrogen, Carlsbad, CA). Analysis of human *CDKN1A* (p21) and *MDM2* was carried out with validated TaqMan Gene Expression Assays specific PCR primers sets (Invitrogen).All samples were run in triplicate using the real time thermal analyzer Rotor-Gene™ 6000 (Corbett, Cambridge, UK), as previously described[25]. Expression values were normalized to the housekeeping gene *POLR2A* amplified in the same sample.

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Keywords: (-)-Nutlin-3 • Thiourea catalyst • *Meso*-diamine desymmetrization • p53/MDM2 • Anticancer agents.

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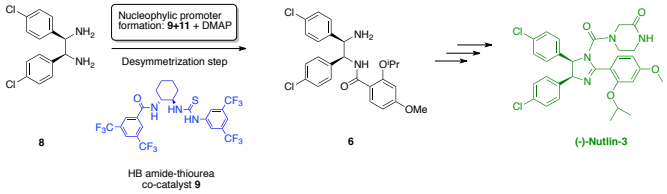

Insert text for Table of Contents here. Make the difference: Starting from easy to make building blocks the synthesis of enriched scalemic mixture of (-)-Nutlin-3 has been accomplished. The use of HB amide thiourea catalyst for the desymmetrization of *meso*-diamine derivative furnished different enantiomers depending on the nature of the acylating agent.