Identification of the first enantiopure Rac1-Tiam1 Protein-Protein interaction inhibitor and optimized synthesis via phosphine free remote group directed hydroarylation

Alessandro Ruffoni [a*], Nicola Ferri [b], Andrea Pinto [c], Sara Pellegrino [d], Alessandro Contini [d], Francesca Clerici [d*]

Phospine free hydroarylation reaction applied to norbornene derivatives is described for the first time and was exploited for the regioselective gram scale synthesis of AR-148 a known Rac1-Tiam1 PPI inhibitor. Umpolung conversion of nitro group to the free amine allowed the regiocontrol of the key arylation step via long range effect. Pharmacological evaluation of the two enantiomers showed the enantioselectivity of the inhibitor and represents the first study case of enantiopure inhibitor of Rac1-Tiam1 PPI

Introduction

The small GTPase protein Rac1 has gained attention for its role in different pathologies, among which cancer and cardiovascular disease [1]. Rac1 regulates events such as smooth muscle cell (SMC) migration [2] and proliferation, [3] and leukocyte-endothelial cell interaction [4]. Rac1 activity depends on the equilibrium between the inactive GDP-bound and the active GTP-bound forms. This cycling is regulated by the guanine nucleotide exchange factors (GEFs) which act as activators, and the GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) which act as negative regulators. The T-cell lymphoma invasion and metastasis 1 (Tiam1) protein, a specific GEF for Rac1, is crucial for cell-cell adhesion and cell migration.

Small molecules interfering with the Rac1-Tiam1 Protein-Protein Interaction (PPI) were identified and reported by us [5-6] and by others [7-11]. (Figure 1a) Afterwards we reported the identification of 2-amino-3-(phenylsulfanyl)norbornane-2-carboxylate as a privileged scaffold for the de novo design and synthesis of a structurally original family of Rac1 inhibitors [12]. (Figure 1b)

G-LISA assay on SMCs demonstrated that among all the compounds tested, **1** (named **AR-148**) selectively and potently inhibits Rac1 without interfering with RhoA. Boyden chamber chemotaxis assay and Cell movement video microscopy analysis have shown how **1** (**AR-148**) affects cell migration in response to the chemotactic agent platelet derived growth factor BB (PDGF-BB) [12].

In our previous work we designed and performed a divergent synthetic strategy from which the entire class of aryl 2-amino-3-(phenylsulfanyl)norbornane-2-carboxylate was obtained.

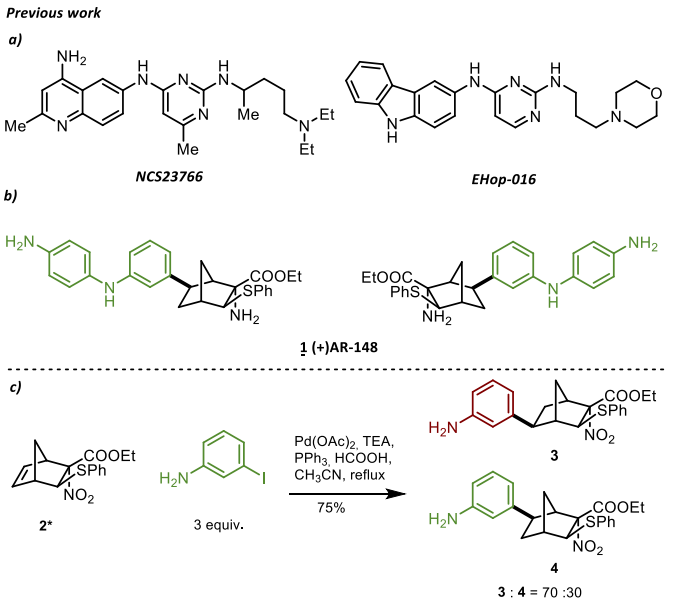

Figure 1. Previous work: a) First generation of Rac-Tiam1 inhibitors; b)Target compound AR-148 obtained in racemic form; c) **AR-148** synthesis drawbacks: -compound **4**, precursor of **AR-148**, is the minor reaction product, - **2*** used as racemate of the two enantiomers

The main difference between **AR-148** and the others known Rac1-Tiam1 PPI inhibitors (Figure 1a, 1b) consists in the presence of a three dimensional core heavily functionalized that places aromatic groups involved in π-π interactions or π-cation interactions in very precise way. (Figure 2)

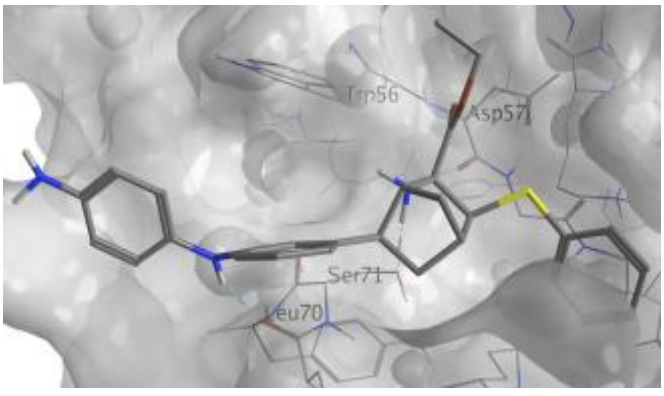

Figure 2. **AR-148** Molecular interaction surface of Rac1-**AR-148 [12]**

[a] School of Chemistry, University of Manchester, Manchester, M13 9PL, UK. E-mail: alessandro.ruffoni@manchester.ac.uk
[b] Department of Pharmaceutical and Pharmacological Sciences, Via Marzolo 5, 35131 Padua, Italy.
[c] Department of Food, Environmental and Nutritional Sciences (DeFENS), Via Mangiagalli 25, 20133 Milano, Italy.
[d] Department of Pharmaceutical Sciences, General and Organic Chemistry Section "Alessandro Marchesini", Via Venezian 21, 20133 Milano, Italy. E-mail francesca.clerici@unimi.it

† Footnotes relating to the title and/or authors should appear here.
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In modern drug discovery, compounds with three dimensional structure that escape the ''flatland'' of multiple arenes, became more central because of higher level of insaturation and the presence of multiple stereocenters. The resulting structural complexity seems to provide better receptor ligand interaction with consequent improved potency, selectivity and decreased off-target effect [13].

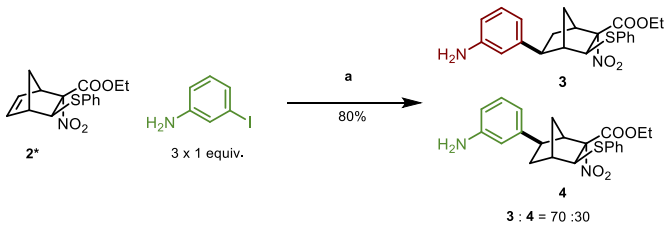
Focusing our attention on the efficient preparation of the most active compound **AR-148** we identified two main drawbacks.

Firstly, the hydroarylation reaction was performed in a classical way in the presence of Ph_3P and excess of iodoarene (3 equiv.) and gave the C-6 arylated isomer, precursor of **AR-148**, as the minor isomer (**3**:**4**=70:30) [14]. (Figure 1c) Secondly, compound **AR-148** was obtained in racemic form. Nowadays, defining the activity of both enantiomers is extremely important because of the great impact on reducing unexpected toxicity in the further drug development. Moreover, once defined the eutomer, its precise pharmacophore model could deeply improve the rational design of new derivatives. As a consequence, the preparation of the two enantiomers in pure form is crucial to evaluate their activity independently. In this work we report an efficient protecting group free, gram scale selective synthesis of compound **AR-148,** the obtainment, via chromatographic separation, of both enantiomers and their pharmacological evaluation.

Results and discussion

Chemistry.

To optimize the synthesis of **AR-148** in an eco-friendly way, we reasoned that a phosphine-free hydroarylation protocol could be of outstanding interest. Many phosphine ligands are expensive, toxic, unrecoverable and often water- and air-sensitive. In large-scale applications on industrial and semi-industrial scale, the phosphines might be a serious economic burden. Therefore we considered performing the reaction in absence of phosphine [15]. To our knowledge phosphine-free Heck hydroarylation has never been realized on olefins and the only examples reported are limited to alkyne substrates [16,17]. So we planned to perform the hydroarylation using the same conditions described before, but in absence of the phosphine ligand ($Pd(OAc)_2$/TEA/HCOOH in CH_3CN). The progressive addition to racemate **2** of 3-iodoaniline (1 equiv. x 3 every 4h) at lower temperature (50°C instead of 82°C) gave the same mixture of regioisomers **3** and **4** in higher yield (80% instead of 75%) but with the same ratio. (Scheme 1)



Scheme 1. Phosphine free methodology. Reagents and conditions: (a) $Pd(OAc)_2$ 5%, TEA 3.5 equiv., HCOOH 3.0 equiv., CH_3CN, 50°C, (80%) *) used as racemate of the 2 enantiomers

The theoretical work done by M.B. Hall on the mechanism of ligand-free Heck type arylation [18-20] and the works published by Cacchi on phosphine-free Heck hydroarylation on alkyne, [16,17] shed light on the different possible scenarios regarding the mechanism of our reaction. Unfortunately, the presence of a slight excess of an organic base like TEA, the use of a coordinative solvent such as CH_3CN, the requirement of formic acid and the substrate itself, containing the norbornene core (ligand in the Catellani reaction [21]), make very difficult to prove which catalytic cycle is really operating or if there are more than one operating at the same time. It is worthy to report that different solvents have been screened (DMF, DMSO, CH_3CN, DCM, MeOH, THF, DCE, Et_2O) and have always led, even if with variable yield, to the formation of the desired product. (Figure 1, SI) The good conversion obtained in DCE underlined how the reaction works, even in the absence of a coordinative solvent, in accordance with the formation of dianionic species isolated by Hartwig and co-workers for the ligand-free Heck arylation [19]. Once a fairly satisfactory result was obtained which demonstrated the feasibility of a phosphine free hydroarylation protocol, we turned our attention to the selectivity of the Heck hydroarylation that is in fact the divergent point in the synthesis of **4**, precursor of the desired compound **AR-148**. In order to modulate the regioselectivity in favour of **4** instead of **3**, several attempts were performed by using different reaction conditions but without satisfactory results [22- 24]. (Table 1, SI)

Our previous studies [14, 25] disclosed that the regioselectivity is mainly driven by long range effect exerted by the nitro group on C-2, (Path a, Figure 3) [26, 27]. We thus decided to exploit the Umpolung conversion of the nitro- to the amine group reasoning that an opposite long range effect should induce an inverse charge distribution of the double bond and consequently an opposite regiochemistry could be obtained (Path b, Figure 3).

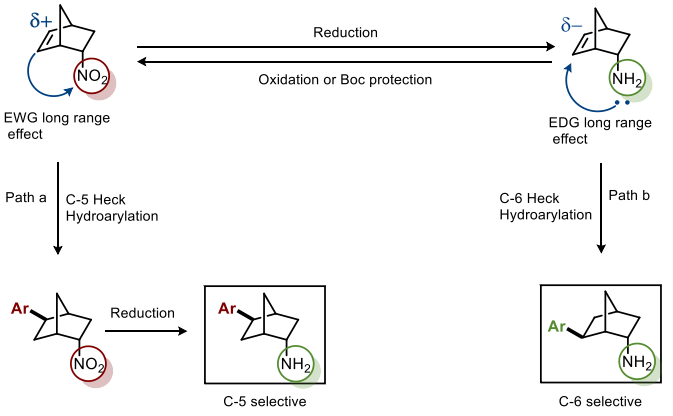
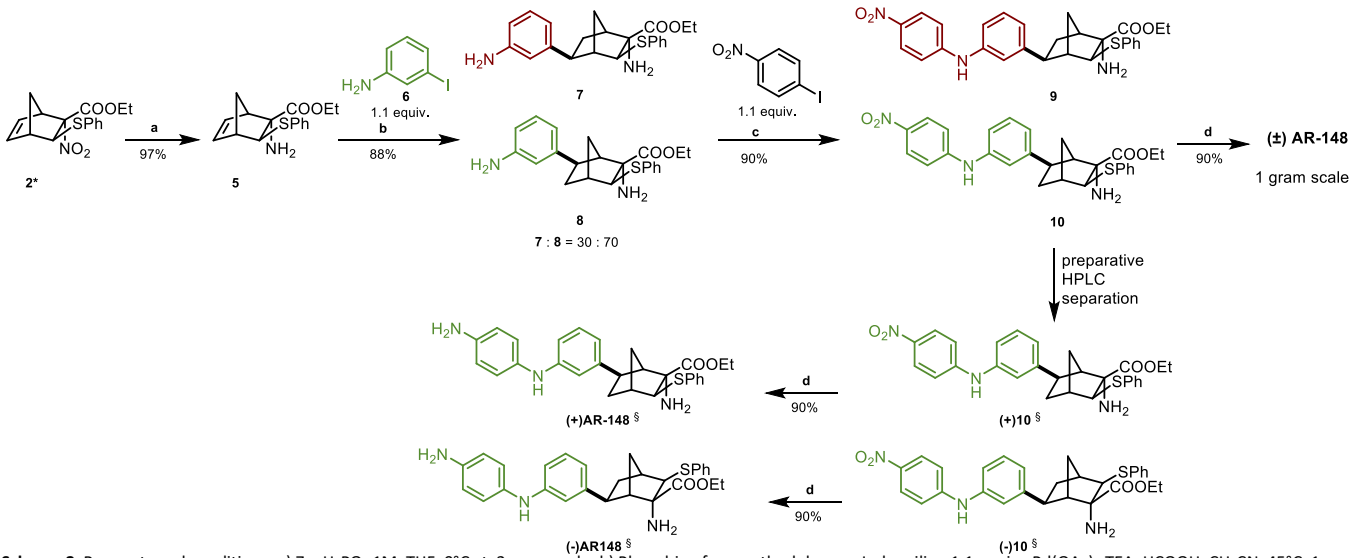


Figure 3. Umpolung conversion of nitro- to amino group to modulate the long range effect on the Hydroarylation reaction

By treating compound **2** with Zn/H_3PO_4 in THF from 0 °C to r.t., amine precursor **5** has been prepared in very good yield (97%). (Scheme 2)

After a brief optimization (Table 2 SI) to identify the adequate reaction conditions compound **7** and **8** have been obtained (yield 88%, 82% for the grams scale) by reacting **5** under the phosphine-free protocol, previously optimized, ($Pd(OAc)_2$/TEA/HCOOH in CH_3CN).



Scheme 2. Reagents and conditions: a) Zn, H_3PO_4 1M, THF, 0°C-rt, 2 gram scale; b) Phosphine free methodology *m*-Iodoaniline 1.1 equiv., $Pd(OAc)_2$,TEA, HCOOH, CH_3CN, 45°C, 1 gram scale; c) 4-Iodonitrobenzene 1.1 equiv., Pd(tetrakis), K_2PO_4, CH_3CN, 70°C, 1 gram scale; d) Zn, HCl 1M, MeOH, 0 °C, 1h, 1 gram scale; *) used as racemate of the 2 enantiomers §)absolute stereochemistry of compounds (-)-10, (+)-10, (-)-AR148 and (+)-AR148 has not been attributed .

As expected thanks to an opposite long range effect, an inverse C5/C6 ratio was found being the regioisomer **8**, precursor of **AR-148**, the main product of the reaction (**7** : **8** = 30 : 70).

Coordination of palladium by the free amine in endo position of **5** can not been excluded but is a non-productive pathway for the Heck Hydroarylation reaction, in fact no endo-product has been detected on norbornene core. [28-30] (See SI page S7 for NMR characterization and assignment of exo-substituted compounds **7** and **8**)

The use of phosphine free protocol has been fundamental to obtain the desired compound as single reaction product and to improve the atom economy of the process. Indeed the undesired Buchwald-Hartwig reaction of *m*-iodo aniline on itself or on the norbornene aliphatic amine of **5** does not work in absence of phosphine while the Heck arylation is not affected. Undesired derivative of **5** is completely suppressed and the amount of *m*-iodo aniline **6** is reduced from 3.0 to 1.1 equivalents. Polymers of *m*-iodo aniline were in fact always isolated using the previous reaction conditions. (Table 2 SI)

Buchwald-Hartwig amination has been tested directly on the inseparable mixture of **7** and **8**. Using Pd(tetrakis), K_3PO_4 and 1.4 equiv. 4-iodonitrobenzene in MeCN, compounds **9** and **10** were obtained in almost quantitative yield (90%). K_3PO_4 is required instead of Cs_2CO_3 to avoid the arylation of the aminoester functionality on norbornene core. The compounds **9** and **10** have been separated by flash chromatography and structure was confirmed by NMR analysis. (Structural elucidation SI). By performing the reduction of the nitro group of **10** with Zn/HCl 1M in MeOH at 0 °C, compound **(±)AR-148** was obtained in a very efficient way. The whole protocol was scaled up and 1 gram of compound **(±)AR-148** was obtained without the use of any protective group, higher atom economy, higher overall yield and in a regioselective way avoiding the use of phosphine in the Heck reaction. (Scheme 2)

Finally, we faced the problem related to the availability of the two enantiomers of **AR-148** for the pharmacological tests. We focused on the enantioselective synthesis of norbornene scaffold **2** obtained through the Diels-Alder cycloaddition reaction [12]. We tried to perform the reaction by using chiral catalysts, but although we observed an enhanced selectivity, very low yields always affected our results. The synthesis of chiral dienophiles (β-sulfanyl nitroacrylate esters of (-)menthol and (-)8-Ph-menthol) was very troublesome and the yields were not suitable as a first step of the synthesis. Therefore, we considered the exploitation of a removable chiral auxiliary to obtain the separation of the two corresponding diastereoisomers of **AR-148**. By reacting the free amine of **AR-148** with (-) menthylchloroformate the two corresponding monomenthylcarbamate diastereoisomers were obtained and easily separated by HPLC, but unfortunately the further hydrolysis of the chiral auxiliary always led to unreacted compounds or tarry materials.

Finally, through the use of chiral HPLC we separated the two enantiomers of **AR-148** with unsatisfactory results. However, excellent results were obtained in the chiral HPLC resolution of racemate **(±)-10** (see SI), (Scheme 2). The two enantiomers **(-)-10** and **(+)-10** were obtained with high enantiomeric purity (purity: >99% ee: >99).

After reduction of the single enantiomers (Zn/HCl in MeOH at 0 °C) the corresponding amines **(-)AR-148** and **(+)AR-148** were obtained. NMR analyses were performed confirming the structure and the purity of the final compounds.

Pharmacology

The effect of **AR-148** and its enantiomers on Rac1 activation was investigated in human cultured smooth muscle cells (SMCs). Interestingly, the **(-)AR-148** enantiomer reduced the intracellular levels of Rac1-GTP with an IC50 value equal to 5.8 µM, while the **(+)AR-148** was inactive. Accordingly, the racemate **AR-148** inhibited the Rac1 activity with an IC50 of 21.7µM. These data clearly demonstrated an enantioselective effect of **(-)AR-148** on Rac1. (Figure 4, Experimental SI)

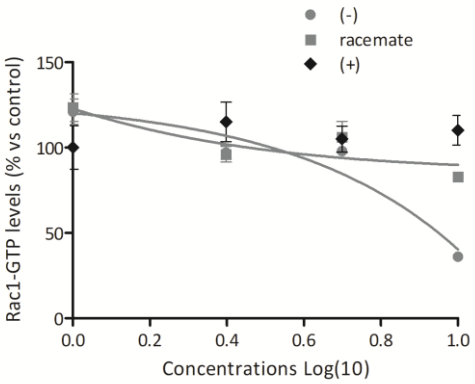


Fig. 4. Dose-dependent effect of **AR148** and its enantiomers on Rac1-GTP levels. SMCs were seeded at a density of 2×105/35 mm Petri dish and incubated with DMEM supplemented with 10% FCS; 24 h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48 h. At this time, the compounds were added to the cultured medium at a final concentration of 1, 2.5, 5 and 10 µM, and after 4 h Rac activation was induced by PDGF-BB (20 ng/mL) for 2 min. Total protein extracts and G-LISA assays were then performed.

Conclusions

In summary we reported a gram scale, protecting group-free synthesis of compound **AR-148**, a known potent inhibitor of Rac1-Tiam1 protein protein interaction. The synthetic strategy applied, includes the first example of phosphine free Heck hydroarylation reaction on an olefin which represents an improvement also in term of eco-friendly and atom economy of the process. We were able to exploit the remote directing group effect of the free amine for the selective obtainment of the desired regioisomer of the arylation reaction.. Despite the impossibility of an enantiopure synthesis, we separated and purified the two enantiomers of **AR-148** and we demonstrated for the first time the enantioselectivity in the inhibition of Rac1-Tiam1 PPI by **(-)-AR-148**. Characterization of the absolute stereochemistry of **(-)AR-148** and study of cocrystalization with the Rac1 protein are proceeding in order to define the pharmacophoric model for a further drug design.

Conflicts of interest

The authors confirm that this article content has no conflict of interest.

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