MedChemComm

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/medchemcomm

MedChemComm

ARTICLE

Integrating Multicomponent Flow Synthesis and Computational Approaches for the Generation of a Tetrahydroquinoline Compound Based Library

Received 00th October 2015,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Bruno Cerra,[a] Serena Mostarda,[a] Chiara Custodi,[a] Antonio Macchiarulo[a] and Antimo Gioiello[a,*]

The combination of flow chemistry and computational tools has been successfully applied to prepare a focused library of tricyclic tetrahydroquinolines endowed with drug-like properties. The study illustrates the efficient synthesis of this class of compounds using flow mesoreactors in a multicomponent fashion, as well as the profitable employment of computational chemistry for library diversity analysis. Early biological characterizations of selected compounds of the library are also presented for the discovery of novel chemical probes to unravel estrogen receptor signaling pathways.

Introduction

The continued identification of novel biological targets combined with readily available high-throughput screening assays require a constant supply of drug-like compounds to enter into the screening process. To satisfy this demand, medicinal chemists are being asked to maximize the coverage of the chemical space more quickly *via* synthetic routes designed to generate diverse small molecules with lead-like or drug-like molecular properties.[1-3] On the one hand, computational approaches have been developed to assess the compliance of compound libraries to drug-likeness concepts with the definition of specific molecular properties associated to the probability of successful development of lead compounds into drug candidates.[4-7] On the other hand, synthetic methods need to be versatile to guarantee the rapid preparation of chemical libraries for the 'hit identification' and 'hit-to-lead optimization' and, at the same time, reproducible to support the large scale synthesis of selected compounds to be advanced into *in vivo* efficacy and safety testing.[8]

To address these issues, in recent years medicinal chemists have experienced a significant evolution in both the mentality and strategies by merging the comprehensive knowledge and creativity in organic synthesis with enabling technologies.[9-11] Complex methodologies and innovative chemical tools such as combinatorial chemistry, nanotechnology, flow and microwave synthesis, biocatalysts, computer-assisted drug design and analysis, automation and engineering are the current arsenal at the service of a chemist. Among these, continuous flow chemistry is gaining ground as one of the techniques that may improve the synthetic and medicinal chemistry sector of drug discovery in terms of efficiency, automation, quality and safety standards, cost-effectiveness and environmental impact.[9,12-14] The potential advantages of flow-based approaches have been exploited in the development of robust and reliable conditions for existing or novel chemical transformations leading to the conduction of safer and greener syntheses, the rapid building of compounds libraries, and the easy scale-up of target compounds.

As part of our interest in the application of flow systems in medicinal chemistry projects,[15-18] in this article we report the results of our efforts aimed at the preparation and early biological characterization of a library of tricyclic tetrahydroquinolines (TC-THQs) endowed with drug-like properties. Novel and straightforward methods for the building of TC-THQ scaffold are greatly sought because of the importance of this nucleus in medicinal chemistry. Indeed, it represents a well-established privileged structure being the common framework of numerous biologically active natural products, pharmaceutically relevant chemical tools and therapeutic agents (Fig. 1).[19] In particular, we have combined the potentiality of the multicomponent Povarov reaction with the advantages of flow chemistry to obtain a THQ-based library readily available for biological screenings. The flow set-up was designed to allow the rapid generation of structurally-related compounds *via* diversification of the heterocyclic core and substituents, as well as to support the multigram-scale preparation of lead candidates. Before library construction and *in silico* computational analysis of the library members, the reaction was optimized in terms of yield and productivity, costs and environmental standpoint. The synthesized compounds were preliminary evaluated as able to occupy the biologically-active chemical space of the G-protein coupled estrogen receptor (GPER)

[a] *Department of Pharmaceutical Sciences, University of Perugia, Via del Liceo 1, I-06122 Perugia (Italy).*
* Corresponding author: e-mail: antimo.gioiello@unipg.it. Tel: +39.075.5852318.
Electronic Supplementary Information (ESI) available: description of the experimental procedures, analytical and spectroscopic characterization of the synthesized compounds, additional biological data. See DOI: 10.1039/x0xx00000x

and then screened for their ability to modulate estrogen signaling pathways using *in vitro* assays.

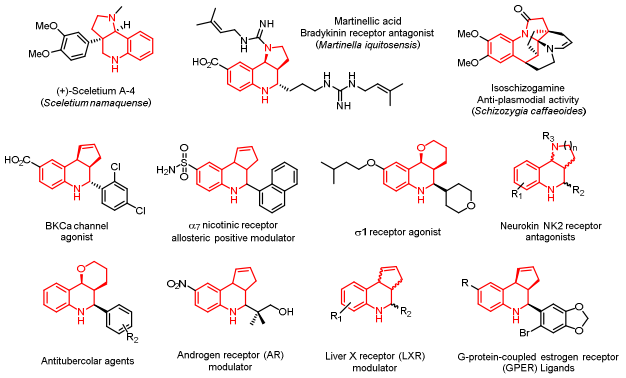


Fig. 1. Representative examples of natural and synthetic tricyclic tetrahydroquinolines of biological interest.

Results and discussion

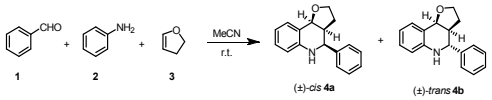
Flow synthesis

Povarov reaction optimization under flow conditions

Povarov reaction is a complexity-generating chemical transformation that has been applied in natural products preparation, diversity-oriented and combinatorial synthesis.[20,21] The reaction permits a wide flexibility in reagents selection allowing both the skeletal, appendage and stereochemical variations. Although initially considered as a formal inverse-electron demand cycloaddition between an electron-rich dienophile and an *in situ* generated *N*-arylimine heterodiene, recent findings proved that the Povarov reaction proceeds by a stepwise mechanism involving the formation and trapping of an ionic intermediate. Arylamines and aromatic aldehydes can be used as the diene components, while cyclic or acyclic vinyl ethers, vinyl enol ethers, vinyl sulfides, enamines, alkenes and alkynes have been employed as electron-rich dienophiles. In batch, the reaction generally proceeds at room temperature in the presence of catalytic amounts (5-30%) of Lewis or Brønsted acids, acidic resins and metal salts.[19-21]

In our case, initial batch screen experiments were performed to select the most appropriate catalyst. Reactions were conducted at room temperature by using equimolar amount of benzaldehyde (**1**), aniline (**2**) and 2,3-dihydrofuran (**3**) as model reaction components, while acetonitrile (MeCN) was the solvent of choice because of its eco-friendly profile and ability to solubilize both reagents and products. Different catalysts were then evaluated for their efficiency on the reaction outcome in terms of reaction time, yield, cost and eco-compatibility (Table 1). As a general trend, most of the catalyst gave high yields though with diverse reaction times. In particular, catalysts employed in entries 4-10 (Table 1) required long reaction times (7-16 h) and were therefore not compatible with the need to translate the process in flow modality. Among the others with favorable reaction time (Table 1, entries 1-3), we selected HCl (10% wt in MeOH) being the cheapest and greenest catalyst.

Table 1. Preliminary batch-screen Povarov reactions[a]



Entry	Catalyst	Amount	Yield[b]/Time
1	$Yb(OTf)_3$	0.1 equiv.	78%/3h
2	$Sm(OTf)_3$	0.1 equiv.	76%/3h
3	HCl (10% wt in MeOH)	0.1 mL $mmol^{-1}$	74%/3h
4	$RhCl_3$	0.1 equiv.	70%/16h
5	$AlCl_3$	0.1 equiv.	50%/16h
6	Amberlist A15/M.S. 4 Å	1 g/100 mg	70%/7h
7	Montmorollenite KSF	1 g	73%/7h
8	Dowex HCR W2	1 g	53%/16h
9	10-camphorsulphonic acid	0.1 equiv.	50%/16h
10	Phosphomolibdic acid	0.1 equiv.	72%/7h
11	$Rh(OAc)_2$	0.1 equiv.	10%/16h

[a]*General procedure*: To a solution of benzaldehyde (**1**) (1 mmol) in MeCN (3 mL), aniline (**2**) (1 mmol), 2,3-dihydrofuran (**3**) (1 mmol) and the catalyst were sequentially added and the resulting mixture was stirred at room temperature till the reaction showed no further progress. [b]Isolated yield after automated flash chromatography. The percentage refers to the diastereoisomeric mixture.

The reaction was then translated into our flow apparatus equipped with loop injection systems, three pumps, a 10 mL reactor-coil, a back pressure regulator (BPR, 100 psi), an UV detector and a fraction collector. During the screening of the experimental conditions, the reactions were sequentially performed by injection of three stock solutions: *a)* benzaldehyde (**1**) (1 mmol, 0.5 M) and HCl (10% wt in MeOH) (loop A, 2 mL), *b)* aniline (**2**) (1 mmol, 0.5 M) (loop B, 2 mL) and *c)* 2,3-didydrofuran (**3**) (loop C, 4 mL).[‡] The flow stream was generated by pumping a solution of 5% (v/v) of polyethylene glycol (PEG) 300 in MeCN. The use of PEG300 as co-solvent was sought to prevent the precipitation of the aniline chlorohydrate within the lines. After the injection, the solutions were mixed in a four way-connector and flowed through the reactor-coil thermostated at 25 °C. The output was monitored by in-line UV detector and collected into a fraction collector (Fig. 2).

We initially focused our investigation by exploring the effect of both flow rate and HCl amount (Table 2). As a result, we observed that high flow rates and strong acidic media were unfavorable for the reaction yield. In particular, HCl might be responsible for the partial, albeit minimal, degradation of the dienophile **3**. Accordingly, a slight excess of 2,3-dihydrofuran (**3**) was beneficial for the reaction outcome leading to an increase in product yield (Table 2, entry 7). Remarkably, the variation of reaction conditions had no significant effect on the *cis/trans* product ratio (*data not shown*). The best result was achieved when the reaction was performed

using 1 equiv. of **1** and **2**, 1.1 equiv. of **3**, 0.2 mL mmol^{-1} of HCl (10% wt in MeOH) and a total flow rate of 0.8 mL min^{-1} (Table 2, entry 7).

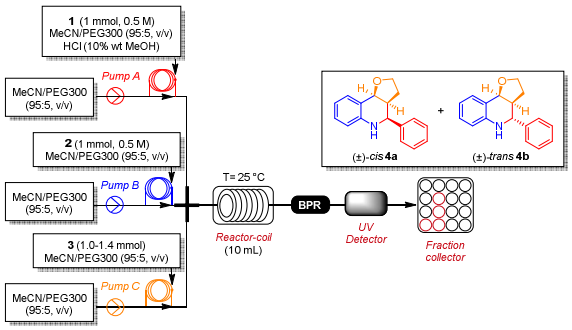


Fig. 2. General flow set-up employed during the reaction optimization.

Table 2. Evaluation of flow rate and catalyst amount on Povarov reaction[a]

Entry	Flow rate (mL min^{-1})[b]	Catalyst amount (mL mmol^{-1})	Equiv. of 3	Yield[c]
1	0.6	0.1	1.0	69%
2	0.8	0.1	1.0	68%
3	1.0	0.1	1.0	56%
4	1.2	0.1	1.0	48%
5	0.8	0.2	1.0	74%
6	0.8	0.4	1.0	58%
7	0.8	0.2	1.1	78%
8	0.8	0.2	1.4	78%

[a]All reactions were conducted according to Fig. 2. [b]Combined flow rate (MeCN/PEG300): pump A (0.2 mL min^{-1}) + pump B (0.2 mL min^{-1}) + pump C (0.4 mL min^{-1}). [c]Determined after silica gel flash chromatography. The percentage refers to the diastereoisomeric mixture.

Library preparation

Our strategy for building the library involved the variations of the three Povarov reaction components in order to diversify product type and prove the reliability and robustness of the methodology. Using the optimized experimental conditions, the reaction was thus applied to *p*-substituted benzaldehydes (**1**, **5**, **6**) and anilines (**2**, **7**, **8**), and different dienophiles characterized by five- and six-member rings (**3**, **9**-**11**) (Table 3). Moreover, the flow equipment was improved with an in-line work-up and automatic purification (Fig. 3). In particular, the reactor output was combined with a stream of H_2O (1.2 mL min^{-1}) and Et_2O (0.4 mL min^{-1}) to facilitate by extraction the removal of salts and PEG300. The ethereal solution was concentrated and submitted to automatic silica gel flash chromatography assisted by UV detector for fraction collection. Reactions were performed in a sequential flow-through process washing the lines with MeCN. Each isolated compound was racemic but diastereomerically pure with the exception of compounds **15**, **17**, **23** and **25**.

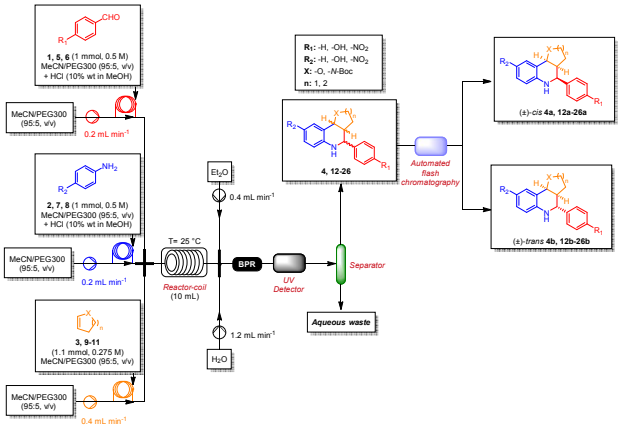


Fig. 3. Optimized flow set-up up for the synthesis of the tricyclic tetrahydroquinoline library.

The assignment of the correct relative configurations was based on the scalar coupling constant between protons H-4 (or H-5) and H-3a (or H-4a) as well as on the evaluation of both chemical shift and multiplicity value of H-9b (or H-10b) (Fig. 4).[22] Thus, in the case of *cis* furano derivatives **4a**, **12a**-**17a**, H-4 showed a small coupling constant (δ= 4.6-4.8 ppm, d, $J_{H\text{-}4/H\text{-}3a}$= 2-4 Hz) typical for a *cis* orientation, while the proton H-9b appeared as a doublet (δ= 5.1-5.3 ppm, d, $J_{H\text{-}9b/H\text{-}3a}$= 7-9 Hz) (Fig. 4). In *trans* furanes **4b**, **12b**-**17b**, protons H-4 and H-9b were slightly shifted downfield (H-4: δ= 3.6-3.9 ppm, d, $J_{H\text{-}4/H\text{-}3a}$= 8-12 Hz; H-9b: δ= 4.4-4.6 ppm, d, $J_{H\text{-}9b/H\text{-}3a}$= 4-6 Hz). Similar values were observed for the pyrano derivatives **18a** (H-5: δ= 4.6-4.7 ppm, d, $J_{H\text{-}5/H\text{-}4a}$= 2-3 Hz; H-10b: δ= 5.2-5.4 ppm, d, $J_{H\text{-}10b/H\text{-}4a}$= 5-6 Hz) and **18b** (H-5: δ= 4.6-4.8 ppm, d, $J_{H\text{-}5/H\text{-}4a}$= 10-12 Hz; H-10b: δ= 4.2-4.4 ppm, d, $J_{H\text{-}10b/H\text{-}4a}$= 2-4 Hz) (Fig. 4). Moreover, the proton H-4 of derivatives **19a**-**25a** and the proton H-5 of **26a** were determined as a doublet of doublet (J_1= 45-65 Hz, J_2= 4-8 Hz), while a broad singlet at 5 ppm was characteristic for the parent *trans* isomers **19b**-**25b** and **26b** (Fig. 4).[23]

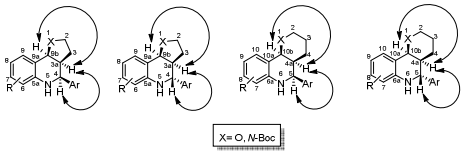


Fig. 4. Assignment of the compound correct relative configuration.

A deeper analysis of the *cis*/*trans* ratios suggested a close correlation between the diastereoselectivity index of the reaction and the dienophile employed. In particular, it was likely that dihydropyran (**9**) and *N*-BOC-dihydropiridine (**11**) provided *trans* isomers as main products (Table 3, entries 8 and 16), while *cis* isomers were favored with five-membered dienophiles **3** and **10** (Table 3, entries 1-7, 9-15). Moreover, *O*-containing dienophiles **3** and **9** (Table 3, entries 1-8) resulted in a higher diastereomeric excess with respect to *N*-containing dienophiles **10**, **11** (Table 3, entries 9-16). As an additional observation, the presence of electron withdrawing groups like NO_2 in the imine component allowed a

higher diastereoselectivity (Table 3, entries 2, 4, 10, 12) while, on the contrary, the presence of electron donating groups as OH reduced the diastereoselectivity ratio (Table 3, entries 3, 6, 11, 14).

Table 3. Flow synthesis of tricyclic tetrahydroquinolines[a]

Entry	Compound	R_1	R_2	X	n	Conversion yield (%)[b]	Isolated yield (%)[c]
1	**4**	-H	-H	O	1	84	65 (**4a**) 13 (**4b**)
2	**12**	$-NO_2$	-H	O	1	92	84 (**12a**) 5 (**12b**)
3	**13**	-OH	-H	O	1	63	40 (**13a**) 18 (**13b**)
4	**14**	-H	$-NO_2$	O	1	85	66 (**14a**) 13 (**14b**)
5	**15**	-OH	$-NO_2$	O	1	88	82 (**15a**:**15b**=78:22)[d]
6	**16**	-H	-OH	O	1	69	39 (**16a**) 25 (**16b**)
7	**17**	$-NO_2$	-OH	O	1	72	66 (**17a**:**17b**= 75:25)[d]
8	**18**	-H	-H	O	2	78	10 (**18a**) 64 (**18b**)
9	**19**	-H	-H	*N*-Boc	1	70	41 (**19a**) 25 (**19b**)
10	**20**	$-NO_2$	-H	*N*-Boc	1	78	58 (**20a**) 16 (**20b**)
11	**21**	-OH	-H	*N*-Boc	1	52	24 (**21a**) 22 (**21b**)
12	**22**	-H	$-NO_2$	*N*-Boc	1	73	53 (**22a**) 15 (**22b**)
13	**23**	-OH	$-NO_2$	*N*-Boc	1	57	52 (**23a**:**23b**= 63:37)[d]
14	**24**	-H	-OH	*N*-Boc	1	54	27 (**24a**) 22 (**24b**)
15	**25**	$-NO_2$	-OH	*N*-Boc	1	69	64 (**25a**:**25b**= 68:32)[d]
16	**26**	-H	-H	*N*-Boc	2	67	15 (**26a**) 47 (**26b**)
17	**27a**[e]	-H	-H	NH	1		34[f]
18	**28a**[e]	$-NO_2$	-H	NH	1		47[f]
19	**29a**[e]	-OH	-H	NH	1		18[f]
20	**30a**[e]	-H	$-NO_2$	NH	1		45[f]
21	**31a**[e]	-H	-OH	NH	1		24[f]
22	**32a**[e]	-H	-H	NH	2		12[f]

[a]All reactions were conducted according to Fig. 3. [b]Determined by ^{1}H-NMR analysis of the reaction mixture. [c]Isolated yield after silica gel flash chromatography. [d]*cis*/*trans* ratio (in brackets) are referred to the diastereomeric mixture after silica gel flash chromatography. [e]Prepared by deprotection of the corresponding (±)-cis *N*-Boc derivatives according to the general procedure reported in the experimental section. [f]Overall yield after*N*-Boc deprotection.

In silico analysis and preliminary biochemical assessments

Evaluation of library drug-likeness

The relationship between physicochemical descriptors and drug-like properties was firstly established by Lipinski's rule-of-five guidelines, with the risk of poorly oral bioavailable compounds increasing with the number of violations to specific values of molecular weight (MW <500), calculated octanol/water partition coefficient (cLogP <5), number of hydrogen bond donor groups (HBD <5) and hydrogen bond acceptor groups (HBA <10).[6] Moreover, topological polar surface area (TPSA) and number of rotatable bonds (RB) were also identified as useful parameters to predict drug-likeness and transport properties.[24-26] Specifically, values of TPSA ≤140 Å^2 and RB ≤10 were suggested for drug-like compounds. Although a strict application of these criteria may introduce artificial distinctions between similar compounds in a chemical library,[27] they are easy to understand allowing a quick identification of how a poor drug-like hit compound could be modified in subsequent hit-to-lead optimization stages.

To assess the drug-likeness of the library, the above molecular descriptors were calculated for all synthesized compounds (Table 4). As a result, library compounds were compliant to the drug-likeness concept, with only one compound showing a single violation of the rule-of-five guidelines due to the slightly higher clogP value (compound **26**, Table 4). With the exception of compounds **23** and **25** (Table 4), the majority of the synthesized heterocycles were also found to be potentially able to cross the blood brain barrier, thereby showing central nervous system activity. Indeed, physicochemical values postulated for small molecules targeting the central nervous system were MW ≤400; clogP ≤5; HBD ≤3; HBA ≤7; TPSA ≤90 Å; RB ≤5.[28]

Table 4. Calculated molecular descriptors[a]

Entry	Compound	Rule-of-5 alerts	MW	cLogP	HBA	HBD	TPSA	RB
1	**4**	0	251.3	3.0	1	1	21.3	1
2	**12**	0	296.3	2.9	1	1	64.4	2
3	**13**	0	267.3	2.7	2	2	41.5	1
4	**14**	0	296.3	2.9	1	1	64.4	2
5	**15**	0	312.3	2.6	2	2	84.6	2
6	**16**	0	267.3	2.7	2	2	41.5	1
7	**17**	0	312.3	2.6	2	2	84.6	2
8	**18**	0	265.3	3.5	1	1	21.3	1
9	**19**	0	350.5	4.6	2	1	41.6	3
10	**20**	0	395.5	4.5	2	1	84.7	4
11	**21**	0	366.5	4.4	3	2	61.8	3
12	**22**	0	395.5	4.5	2	1	84.7	4
13	**23**	0	411.5	4.3	3	2	104.9	4

14	**24**	0	366.5	4.4	3	2	61.8	3
15	**25**	0	411.5	4.3	3	2	104.9	4
16	**26**	1	364.5	5.1	2	1	41.6	3
17	**27**	0	250.3	2.7	0	1	24.1	1
18	**27**[b]	0	251.3	1.5	0	2	28.6	1
19	**28**	0	295.3	2.6	0	1	67.2	2
20	**28**[b]	0	296.3	1.4	0	2	71.8	2
21	**29**	0	266.3	2.5	1	2	44.3	1
22	**29**[b]	0	267.3	1.2	1	3	48.9	1
23	**30**	0	295.3	2.6	0	1	67.2	2
24	**30**[b]	0	296.3	1.4	0	2	71.8	2
25	**31**	0	266.3	2.5	1	2	44.3	1
26	**31**[b]	0	267.3	1.2	1	3	48.9	1
27	**32**	0	264.4	3.2	0	1	24.1	1
28	**32**[b]	0	265.4	2.0	0	2	28.6	1

[a]Compound minimization and generation of tautomers and protonation states (pH 7±2) were performed using LigPrep software (*version 3.2, Schrödinger, LLC, New York,* 2014). The Lipinski's descriptors (MW, clogP, HBA, HBD), the topological polar surface area (TPSA) and the number of rotatable bonds (RB) were calculated using Canvas (*version 2.2, Schrödinger, LLC, New York,* 2014) taking into account the atomic connectivity without considering the 3D arrangement. [b]H^+-form.

Tricyclic tetrahydroquinolines as GPER ligands

TC-THQs were found to interact with different biological targets as nuclear and membrane receptors, enzymes and ion channels, and to exhibit a wide spectrum of biological actions including neuroprotection, androgenic, estrogenic, analgesic, anticancer and antimicrobial (Fig. 1).[19] In particular, it was shown that *cis* tetrahydro-3*H*-cyclopenta[*c*]quinolones are a valuable class of compounds able to bind and activate GPER receptor.[29-31] GPER (formerly GPR30) belongs to the sub-class A of rhodopsin-like GPCRs and it is thought to mediate rapid biological responses to normal estrogen physiology and pathology.[32] In the last years, GPER emerged as an appealing therapeutic target for the treatment of a plethora of diseases such as obesity, diabetes, atherosclerosis, inflammation and cancer despite a number of questions are still open on the exact role of the receptor in these disorders.[33-35]

Based on these considerations and following our interest in steroid-responsive receptors,[36,37] we decided to evaluate the activity of the synthesized products as GPER ligands. Initially the library was analysed within the biologically-active chemical space of known GPER ligands. A principal component analysis (PCA) was built on the molecular descriptors previously calculated (Table 4) and applied to the TC-THQs library members and known GPER ligands, including 17β-estradiol (**33**), fulvestrant (**34**), G15 (**35**), G1 (**36**), G36 (**37**), MIBE (**38**) and tamoxifene (**39**) (Fig. 5).

The first two components explained 95.6% of cumulative variance with MW and TPSA representing the highest loading vectors for the first and the second principal component, respectively. The PCA has unveiled that the synthesized compounds (grey circles) occupy mainly four regions in the GPER chemical space. In particular, compounds **12a**, **14a**, **16a** and **28a**-**31a** are located in the same area occupied by the endogenous agonist 17β-estradiol (**33**) (black square) (Fig. 5, region 3, orange area) while a sort of 'already explored' area is depicted at the fourth quadrant in which are located compounds **19a** and **26a** together with the selective THQ-based GPER selective ligands G15 (**35**) (circle), G1 (**36**) (square), G36 (**37**) (triangle) as well as 4-hydroxytamoxifen (**39**) (cross) (Fig. 5, region 1, light green area). Finally, two additional 'unexplored regions' with several library members but no reported GPER ligands, are situated at the negative values of the first component. In particular, in the region 2 (Fig. 5, light blue area) are located compounds **4a**, **18a**, **27a** and **32a**, while the region 4 (Fig. 5, pale yellow area) contains only the protonation states of *N*-Boc derivatives.

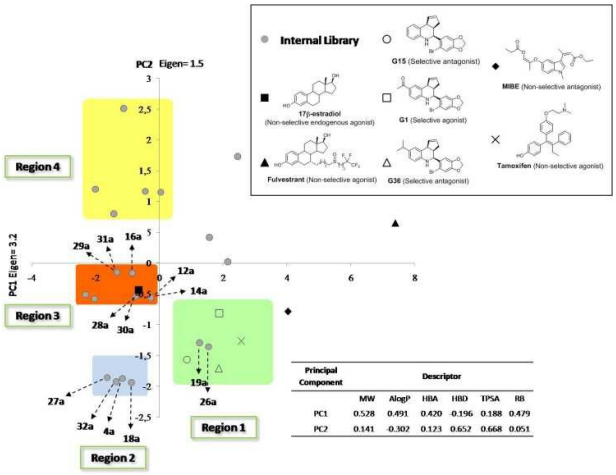


Fig. 5. Tricyclic tetrahydroquinoline library in GPER biologically-active chemical space.

A selection of the synthesized compounds with *cis* relative configuration was thus tested in a PathHunter β-arrestin GPCR assay for assessing their potential GPER agonistic activity (Table 5). Most of the compounds resulted in weaker or no responses in this system, while compound **4a** and **31a** were found to elicit a similar activation to the endogenous ligand 17β-estradiol (**33**) (K_i=2.7-6.6 nM).[38,39] Interestingly, none of these compounds acted as ligands of the estrogen receptor α (ERα) both in agonist and antagonist mode when tested in Alphascreen coactivator assays (Table 5) at three different doses (10, 50 and 100 μM).

Table 5. Preliminary biological activity of selected tricyclic THQs toward GPER and ERα[a]

Entry	Compound	GPER Agonism[b]	ERα (EC_{50})[c]
1	**33**	+++	3 nM
2	**4a**	+++	> 100 μM
3	**12a**	+	> 100 μM
4	**14a**	++	> 100 μM

5	**16a**	+	> 100 μM
6	**18a**	–	> 100 μM
7	**27a**	–	> 100 μM
8	**28a**	–	> 100 μM
9	**29a**	–	> 100 μM
10	**30a**	+	> 100 μM
11	**31a**	+++	> 100 μM
12	**32a**	–	> 100 μM

[a]TC-THQ derivatives were tested as pure *cis* diastereoisomers but as racemates. [b]Activity onGPER was assayed by PathHunter β-arrestin GPCR assay (*DiscoveRx*) and target compounds were tested in a single dose (10 μM) in duplicate in presence of 17β-estradiol (10 μM) as positive reference compound. +++,++, + and - denote that GPER efficacy in response to 10 μM of the target compound is respectively 75-100%, 50-75%, 25-50% and <25% of reference compound (17β-estradiol, 10 μM). [c]Activity on *h*ERα was assayed by Alphascreen technology in a coactivator recruitment assay (Ref.: 17β-estradiol, EC_{50}= 3 nM). Three different doses (10, 50 and 100 μM) were performed and the experiments were run in duplicate.

Conclusions

In this work we have shown the successful combination of flow technologies and computational approaches for the design, synthesis and analysis of a focused THQ-based library endowed with drug-like properties. In particular, integrated flow systems were profitably employed to translate the multicomponent Povarov reaction in a continuous flow modality. The method resulted to be superior to the reported batch protocols affording the desired TC-THQ derivatives from good to high yields. The flow synthesis platform incorporates mesoreactors, in-line work-up and purifications that can be performed under automation reducing material handling and timing while increasing safety standards and production. Although this work primarily focused on TC-THQ scaffold, the general approach and flow set-up described possesses a more widely substrate scope. This work may open the way to highly efficient generation of drug-like heterocycles to support medicinal chemistry programs at various stages from early drug discovery to production.

Remarkably, the synthesized compounds are endowed with drug-like properties and show biological activity in preliminary *in vitro* assays. At this regards, while further biological appraisals are needed to confirm preliminary data and guide future medicinal chemistry investigations, the results so far obtained allow disclosing a novel class of ligands with promising selective GPER activity.

Experimental

General methods

Unless otherwise noted, chemicals were obtained from commercial suppliers and used without further purification. Melting points were determined using a Buchi 535 electrothermal apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC 200 MHz or 400 MHz spectrometer in the indicated solvent. Chemical shifts are reported in parts per million (ppm) and are relative to $CDCl_3$ (7.26 ppm and 77.0 ppm) or d^6-DMSO (2.50 ppm and 39.7 ppm). The abbreviations used are as follows: s, singlet; brs, broad singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; brm, broad multiplet. Flash column chromatography was performed using Biotage Isolera One. Thin-layer chromatography was performed using glass plates coated with silica gel 60 F-254. Spots were visualized by UV detector (λ: 254 nm) and/or by staining and worming with potassium permanganate. All flow experiments were performed using a commercially available Vapourtec R2+/R4 module equipped with two-loop injection systems (2 mL each), two integrated and one external HPLC pumps, a 10 mL PTFE coil-mesoreactor, a back pressure regulator (BPR, 100 psi), an UV detector and a fraction collector. GC-MS analysis were carried out with Agilent 6850 gas chromatograph equipped with Agilent 5975B mass selective detector. Diasteromeric excess and chemical purity were evaluated by chiral HPLC analysis using a Shimadzu (Kyoto, Japan) LC-20A Prominence equipped with a CBM-20A communi-cation bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 20 μL stainless steel loop. A Chiracel OD-H column (250 mm x 4.6 mm, 5 μm, 100 Å) was used as the analytical column. The analyses were carried out at 1.0 mL min^{-1} flow rate after previous conditioning by passing through the column the selected mobile phase for at least 30 min at the same eluent velocity. Before being used, the mobile phases were always filtered through a 0.22 μm millipore filter (Bedford, MA, USA) and then degassed with 20 min sonication. The column temperature was controlled through a Grace (Sedriano, Italy) heather/chiller (Model 7956 R) thermostat. All the analyses were conducted at 25 °C column temperature using a mixture *n*-hexane/*i*-PrOH (99:1 or 95:5, v/v) as mobile phase.

Flow synthesis of tricyclic tetrahydroquinolines

A solution of aniline (**2**, **7** or **8**) (0.5 M, 1 mmol, 2 mL) in MeCN/PEG300 (95:5, v/v) and a solution of aldehyde (**1**, **5** or **6**) (0.5 M, 1 mmol, 2 mL) in MeCN/PEG300 (95:5, v/v), containing HCl (0.2 mL, 10% wt in MeOH) as catalyst, were injected into the loops and pumped with a flow rate of 0.2 mL min^{-1} for each pump. After the injection and switching of the valves into the loops, a solution of the dienophile (**3** or **9**-**11**) (0.275 M, 1.1 mmol, 4 mL) in MeCN/PEG300 (95:5, v/v) was pumped by an external pump with a flow rate of 0.4 mL min^{-1}. The three components were mixed together in a four way-connector and flowed through the reactor-coil thermostated at 25 °C. The output was detected by an in-line UV detector and then combined with a stream of H_2O and Et_2O pumped at 1.2 mL min^{-1} and 0.4 mL min^{-1}, respectively. The combined stream was directed through an in-line separatory funnel and the organic phase was collected, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The reaction crude was analyzed by ^{1}H-NMR (200 Mhz, $CDCl_3$ or d^6-DMSO) to evaluate the *cis*/*trans* ratio and then purified by automatic flash chromatography on silica gel (eluent: petroleum ether/Et_2O from 100:0 to 85:15, v/v, or petroleum

ether/EtOAc from 100:0 to 70:30, v/v) affording pure *cis* and *trans* THQ derivatives **4**, **12**-**26** as racemic mixtures.

General procedure for *N*-Boc deprotection

To a stirred solution of *N*-Boc protected derivatives (0.2 mmol) in dry CH_2Cl_2 (1 mL), cooled to 0 °C, trifluoracetic acid (150 μL, 2 mmol) was added. After 30 min the mixture was allowed to warm to room temperature and stirred for 0.5-2.5 h. The reaction mixture was slowly quenched with aqueous saturated solution of $NaHCO_3$ (5 mL), washed with H_2O (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude was filtered on a short silica pad(eluent: $CHCl_3$/MeOH from 100:0 to 85:15, v/v) and triturated with *n*-hexane affording the desired THQs **27a**-**32a**.

Computational chemistry

LigPrep software (*version 3.2, Schrödinger, LLC, New York,* 2014) was used for the compound minimization and for the generation of tautomers and protonation states (pH 7±2). Chirality was determined by 3D structure taking in consideration only one enantiomer. Lipinski's descriptors (MW, clogP, HBA, HBD), topological polar surface area (TPSA) and the number of rotatable bonds (RB) were calculated for the generated compounds using Canvas (*version 2.2, Schrödinger, LLC, New York,* 2014). The principal component analysis (PCA) was performed with Canvas (*version 2.2, Schrödinger, LLC, New York,* 2014).

Biology

Selected compounds were screened as pure diastereoisomers in their racemate forms. GPER activation was evaluated at *DiscoverRx* (Fremont, CA, USA) by using PathHunter β-arrestin GPCR assay.[40] Cells (CHO) were seeded in a total volume of 20 μL into white walled, 384-well microplates and incubated at 37 °C for the appropriate time prior to testing. For agonist determination, cells were incubated with sample to induce response. Intermediate dilution of sample stocks was performed to generate 5X sample in assay buffer. 5 μL of 5X sample was added to cells and incubated at 37 °C or room temperature for 90 to 180 min. After appropriate compound incubation, assay signal was generated through a single addition of 12.5 μL (50%, v/v) of PathHunter detection reagent, followed by 1 h incubation at room temperature. Microplates were read following signal generation with a PerkinElmer EnvisionTM instrument for chemiluminescent signal detection. In parallel to compound testing, cell lysates were also tested for inducible and constitutive arrestin recruitment by EFC positive and negative controls, respectively. Compound activity was analyzed using CBIS data analysis suite (*ChemInnovation, CA*). Activity was calculated as the percentage increase relative to the basal activity observed in the presence of vehicle using the following formula:

%Activity= 100 x (mean RLU of test sample - mean RLU of vehicle control)/(mean RLU of vehicle control)

Activity against *h*ERα was evaluated by using a recruitment coactivator assay, namely AlphaScreen technology, according to a previously reported protocol,[41] in presence of 17β-estradiol (**33**) (EC_{50}= 3 nM) as positive control. Assay were conducted in white, low-volume, 384-well OptiPlate using a final volume of 25 μL containing glutathione transferase-tagged NR-LBD protein (10 nM) and biotinylated coactivator peptide (30 nM). The stimulation was carried out with 1 μL of each tested compound solubilized in 100% DMSO, for 30 min at room temperature. Luminescence was read in EnVision microplate analyzer after incubation with detection mix (acceptor and donor beads) for 4 h at room temperature in the dark. Three different doses (10, 50 and 100 μM) were performed and the experiments were run in duplicate.

Acknowledgements

We thank TES Pharma (Taverne di Corciano, Perugia, Italy) for the assessment of the activity on ERα receptor, and Daniela Passeri and Francesca De Franco for the technical support and discussion related to the reported biological activity.

Notes and references

‡During the screening of the experimental conditions the concentration of 2,3-didydrofuran (**3**) was varied from 0.25 M (1.0 equiv., 1.0 mmol, 4 mL) to 0.35 M (1.4 equiv., 1.4 mmol, 4 mL).

1 A. Nadin, C. Hattotuwagama and I. Churcher, *Angew. Chem. Int. Ed.*, 2012, **51**, 1114-1122.
2 L. Di, E. H. Kerns and G. T. Carter, *Curr. Pharm. Des.*, 2009, **15**, 2184-2194.
3 C. Cordier, D. Morton, S. Murrison, A. Nelson and C. O' Leary-Steele, *Nat. Prod. Rep.*, 2008, **25**, 719-737.
4 W. P. Walters, A. A. Murko and M. M. Murcko, *Curr. Opin. Chem. Biol.*, 1999, **3**, 384-387.
5 E. J. Martin and R. E. Critchlow, *J. Comb. Chem.*, 1999, **1**, 32-45.
6 C. A. Lipinski, *J. Pharmacol. Toxicol. Methods*, 2000, **44**, 235-249.
7 D. E. Clark and S. D. Pickett, *Drug Discov.Today,* 2000, **5**, 49-58.
8 Janero, D. R, *Exp. Opin. Drug Discov.*, 2014, **9**, 847-858.
9 S. V. Ley, D. E. Fitzpatrick, R. J. Ingham and R. M. Myers, *Angew. Chem. Int. Ed.*, 2015, **54**, 3449-3464.
10 J. G. Lombardino and J. A. Lowe, *Nat. Rev. Drug Discov.*, 2004, **3**, 853-862.
11 S. A. Patil, *Org. Chem. Curr. Res.*, 2012, **1**, 1000e110.
12 M. Baumann, I. R. Baxendale and S. V. Ley, *Mol. Diversity*, 2011, **15**, 613-630.
13 C. Wiles and P. Watts, *Green Chem.*, 2012, **14**, 38-54.
14 J. Wegner, S. Ceylan and A. Kirschning, *Chem. Commun.*, 2011, **47**, 4583-4592.
15 P. Filipponi, C. Ostacolo, E. Novellino, R. Pellicciari and A. Gioiello, *Org. Process Res. Dev.*, 2014, **18**, 1345-1353.
16 S. Mostarda, P. Filipponi, R. Sardella, F. Venturoni, B. Natalini, R. Pellicciari and A. Gioiello, *Org. Biomol. Chem.*, 2014, **12**, 9592-9600.
17 A. Gioiello, E. Rosatelli, M. Teofrasti, P. Filipponi and R. Pellicciari, *ACS Comb. Sci.,* 2013, **15**, 235-239.
18 F. Venturoni, A. Gioiello, R. Sardella, B. Natalini and R. Pellicciari, *Org. Biomol. Chem.*, 2012, **10**, 4109-4115.
19 V. Sridharan, P. A. Suryavanshi and J. C. Menèndez, *Chem. Rev.*, 2011, **111**, 7157-7259, and references cited herein.
20 V. V. Kouznetsov, *Tetrahedron,* 2009, **65**, 2721-2830.
21 V. A. Glushkov and A. G. Tolstikov, *Russ. Chem. Rev.*, 2008, **77**, 137-151.

22 B. H. S. T. da Silva, N. L. Marana, A. C. Mafud and L. C. da Silva-Filho, *Struct. Chem.*, 2014, **25**, 327-337.
23 Patent (Takeda Pharm.), *WO*105802A1, 2005.
24 J. Kelder, P. D. J. Grootenhuis, D. M. Bayada, L. P. C. Delbressinc and J. P. Ploemen, *Pharm. Res.*, 1999, **16**, 1514-1519.
25 D. F. Veber, S. R. Johnson, H. Y. Cheng, B. R. Smith, K. W. Ward and K. D. Kopple, *J. Med. Chem.*, 2002, **45**, 2615-2623.
26 P. Ertl, B. Rohde and P. Selzer, *J. Med. Chem.*, 2000, **43**, 3714-3717.
27 I. Yusof and M. D. Segall, *Drug Discov. Today*, 2013, **18**, 659-666.
28 H. Pajouhesh and G. R. Lenz, *NeuroRx*, 2005, **2**, 541-553.
29 C. Bologa, C. M. Revankar, S. M. Young, B. S. Edwards, J. B. Arterburn, A. S. Kiselyov and M. A. Parker, *Nat. Chem. Biol.*, 2006, **2**, 207-212.
30 M. K. Dennis, R. Burai, C. Ramesh, W. K. Petrie, S. N. Alcon, T. K. Nayak, C. G. Bologa, A. Leitao, E. Brailoiu, E. Deliu, N. J. Dun, L. A. Sklar, H. J. Hathaway, J. B. Arterburn, T. Oprea and E. R. Prossnitz, *Nat. Chem. Biol.*, 2009, **5**, 421-427.
31 M. K. Dennis, A. S. Fied, R. Burai, C. Ramesh, W. K. Petrie, C. G. Bologa, T. Oprea, Y. Yamaguchi, S. Hayashi, L. A. Sklar, H. J. Hathaway, J. B. Arterburn and E. Prossnitz, *J. Steroid Biochem. Mol. Biol*., 2011, **127**, 358-366.
32 H. M. Gaudet, S. B. Cheng, E. M. Christensen, E. J. Filardo, *Mol. Cell. Endocrinol.* 2015, *in press*, doi: 10.1016/j.mce.2015.07.016.
33 M. Barton and E. R. Prossnitz, *Trends Endocrinol. Metab.*, 2015, **26**, 185-192.
34 E. R. Prossnitz and M. Barton, *Mol. Cell. Endocrinol.*, 2014, **389**, 71-83.
35 E. R. Prossnitz and J. B. Arterburn, *Pharmacol. Rev.,* 2015, **67**, 505-540.
36 A. Gioiello, B. Cerra, S. Mostarda, C. Guercini, R. Pellicciari and A. Macchiarulo, *Curr. Top. Med. Chem.*, 2014, **14***,* 2159-2174.
37 A. Gioiello, E. Rosatelli, R. Nuti, A. Macchiarulo and R. Pellicciari, *Expert Opin. Ther. Pat.*, 2012, **22**, 1399-1414.
38 P. Thomas, Y. Pang, E. J. Filardo and J. Dong, *Endocrinol.*, 2005, **146**, 624-632.
39 C. M. Revankar, D. F. Cimino, L. A. Sklar, J. B. Arterburn and E. R. Prossnitz, *Science*, 2005, **307**, 1625-1630.
40 *http://www.discoveRx.com.*
41 G. Rizzo, D. Passeri, F. De Franco, G. Ciaccioli, L. Donadio, S. Orlandi, B. Sadeghpour, X. X. Wang, T. Jiang, M. Levi, M. Pruzanski and L. Adorini, *Mol. Pharmacol.*, 2010, **78**, 617-630.

Graphical Abstract

Integrating Multicomponent Flow Synthesis and Computational Approaches for the Generation of a Tetrahydroquinoline Compound Based Library

Bruno Cerra, Serena Mostarda, Chiara Custodi, Antonio Macchiarulo and Antimo Gioiello*