Objectives.

As described in the Introduction, the challenge of designing receptor-selective compounds that preserve the physiological activity of TRPV1, not compromising the normal sensory signaling, while down-regulating the function of overactive receptors is still open. The preparation of new compounds that allow to develop SAR databases to gain insight on their mechanism of action and the determinants of activity is essential for the development of new TRPV1-based analgesic leads.

With these antecedents, the main objectives planned for this thesis were:

1) Synthesis of collections of new conformationally constrained compounds, based on the structures of the peptoid TRPV1 antagonists DD161515 and DD191515.
2) Biological evaluation of the above compounds using *in vitro* and *in vivo* assays to check their potency as TRPV1 antagonists, their selectivity in front of other ion channels and their toxicity.
3) Generation of a SAR database to conduct 3D-QSAR studies that lead to the development of TRPV1-blockade activity models with predictive capacity.
4) Investigation on the structural determinants of the activity and the mode of action of the newly developed TRPV1 blockers

Chapter 2 - Synthesis of new TRPV1 uncompetitive antagonists.

2.1 Preliminary attempts to restrict the flexibility of the peptoid scaffold.

As stated in the Introduction, at the beginning of this project, the existing data for a relatively small amount of compounds with uncompetitive antagonist activity against TRPV1[60, 72, 74, 75] allowed to propose a simple pharmacophoric hypothesis consisting on:[69,70,83,84]

- A cationic moiety, which could be an alkyl guanidine or a protonable alkyl amine group (Figure 23a), and which was assumed to interact with the negatively charged vestibule of the TRPV1 pore.
- Two aromatic groups that could interact with aromatic or hydrophobic counterparts on the protein (Figure 23b).

Preliminary work carried out in our group (A. Moure, PhD thesis) with the aim of transforming the flexible peptoid scaffold into a more rigid structure that complies with the above pharmacophore, led to the design and synthesis of several diketopyperazine and 1,4-diazepine analogues.[88] This strategy had previously been explored by our group with success. For example, the cyclic peptoid-derived molecule **1** (Figure 25) showed a lower toxicity and higher antiapoptotic activity than the parent compound,[79] where as the cyclic derivatives **2** and **3** were designed to interfere the Ubc13-Uev1 interaction at micromolar concentration.[89]

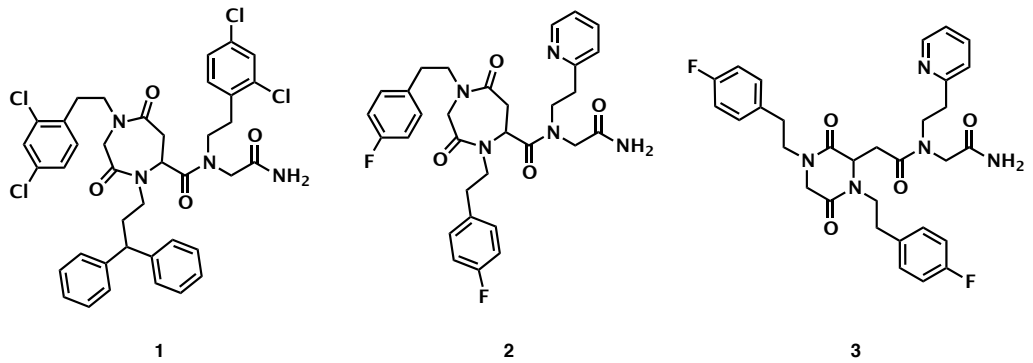


Figure 25. Structures of products evolved from initial peptoid hits that contain the perhydro-1,4-diazepine-2,5-dione (1, 2) and piperazine-2,5-dione (3) scaffolds.[79,89]

Thus, a small collection of cyclic peptoid derivatives (Figure 26) were designed and synthesized using previously described methods,[79] and tested against TRPV1.

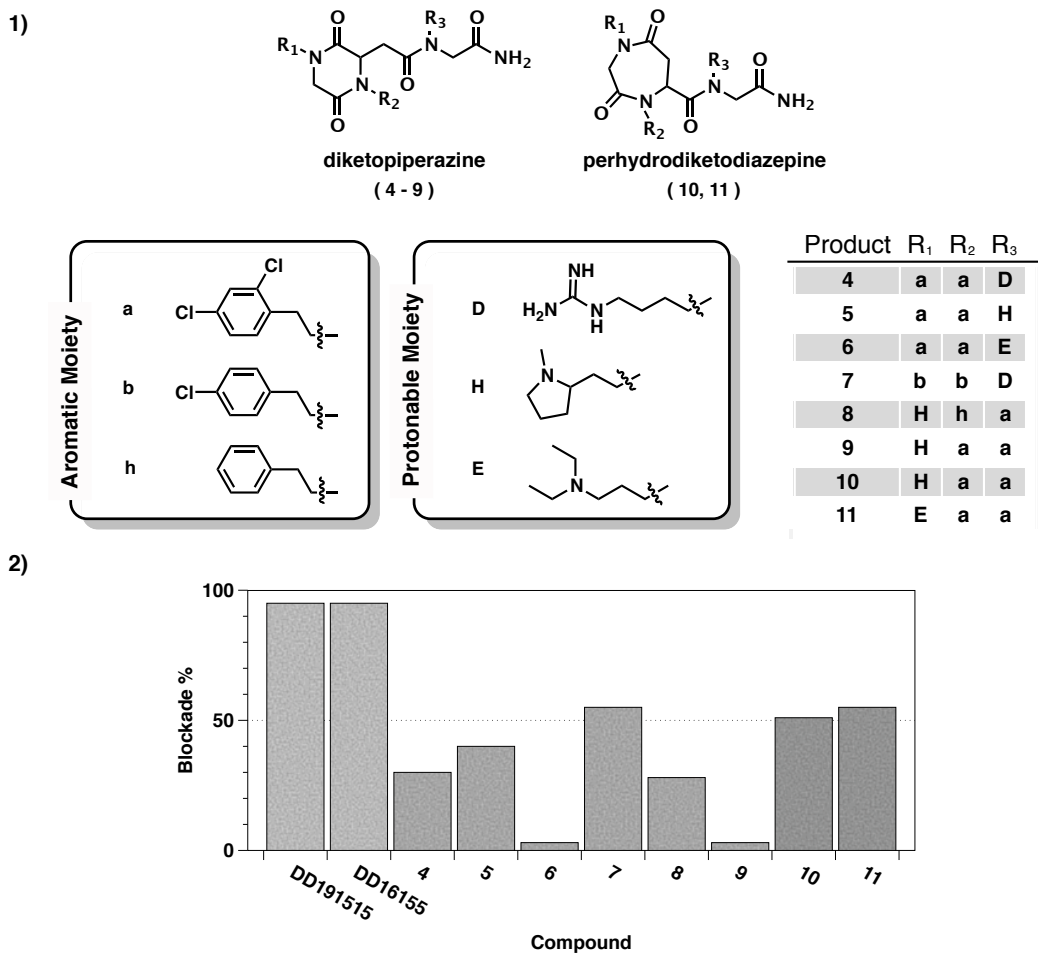
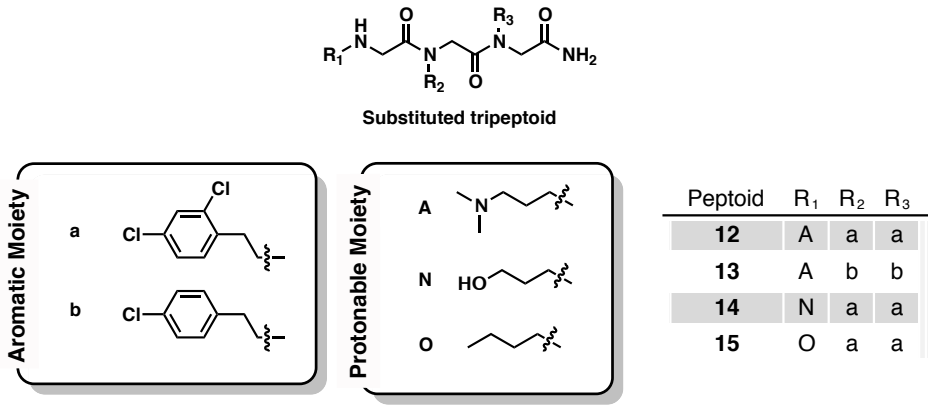


Figure 26: 1) Structures of initially attempted cyclic peptoid derivatives. 2) Blocking potency of initial peptoid hits and cyclic derivatives **4**-**11** assayed at 10 μM.

All synthesized compounds presenting a restricted scaffold exhibited a lower activity than peptoids DD191515 and DD161515. Most products containing the same substitution as the former peptoids DD191515 and DD161515, namely **5**, **10** and **11**, showed about half the blocking potency of the former peptoids. On the other hand, products **6** and **9** were very inactive although containing the same substituents. Guanidine derivatives **4** and **7** exhibited an activity comparable to the active restricted peptoid analogs **5**, **10** and **11**. Altogether, since the TRPV1 antagonist potency elicited by these compounds was markedly lower than that of the original and more flexible peptoid hits (Figure 26), it was reasoned that although satisfying the requirements of the pharmacophore, the suboptimal 3D arrangement of structural features imposed by the diketopiperazine and perhydrodiketodiazepine scaffolds could be responsible for the modest or null activity observed. Furthermore, the structure of these scaffolds still held a relatively high degree of flexibility, and they included chiral centres and three non-equivalent positions for substitution, which render a large number of possibilities if

exploring different combinations of R_1-R_3 was considered. Therefore, it was envisaged that alternative scaffolds had to be explored.

With these antecedents and considering that some aspects of the proposed pharmacophore were not completely proven, namely that compounds lacking the cationic group or the dichlorophenyl moieties would show a decreased potency or be completely devoid of activity, it was initially decided to turn back to the original peptoid scaffold and prepare a few slightly modified compounds to explore those extremes. Hence, we deemed that the synthesis of peptoids where the cationic groups of D161515 and D191515 had been replaced by a polar non-ionic (i.e. hydroxyl), a non-polar (i.e. alkyl), or a different cationic (i.e. dimethylamino) moiety could provide information about the requirement of a positive charge for TRPV1-antagonism. Similarly, replacement of the dichlorophenyl groups by a differently substituted aromatic moiety could also help to define the dependence of the activity on this part of the molecules. Therefore, peptoids **12-15** were pipelined for synthesis (Figure 27).



Peptoid	R_1	R_2	R_3
12	A	a	a
13	A	b	b
14	N	a	a
15	O	a	a

Figure 27. Structures of peptoids **12-15** depicted as a peptoid scaffold and side chain decoration. Table on the right describes peptoid substitution.

2.2 Synthesis of Peptoids 12-15.

Peptoids **12** - **15** were synthesized using the rink amide solid phase methodology previously described by our group (Figure 28),[83] being this a modification of the procedure described by Fligozzi *et al.*[90] To improve yields, decrease impurity levels and accelerate the reactions, microwave activation was used. Individual peptoids were synthesized in polypropylene (PP) syringes with a polypropylene porous plate at the end of the syringe and a teflon cap. Selection of the diversity side chains was made on the assumption that small changes to the structure of the active compounds would

still generate active products. For that reason a 4-chlorophenethyl moiety was selected instead of 2,4-dichlorophenethyl group. On the other hand the protonable side chain was exchanged by 3-dimethylaminopropyl, 3-hydroxypropyl and propyl groups to check for the relevance of the chargeable moiety in activity.

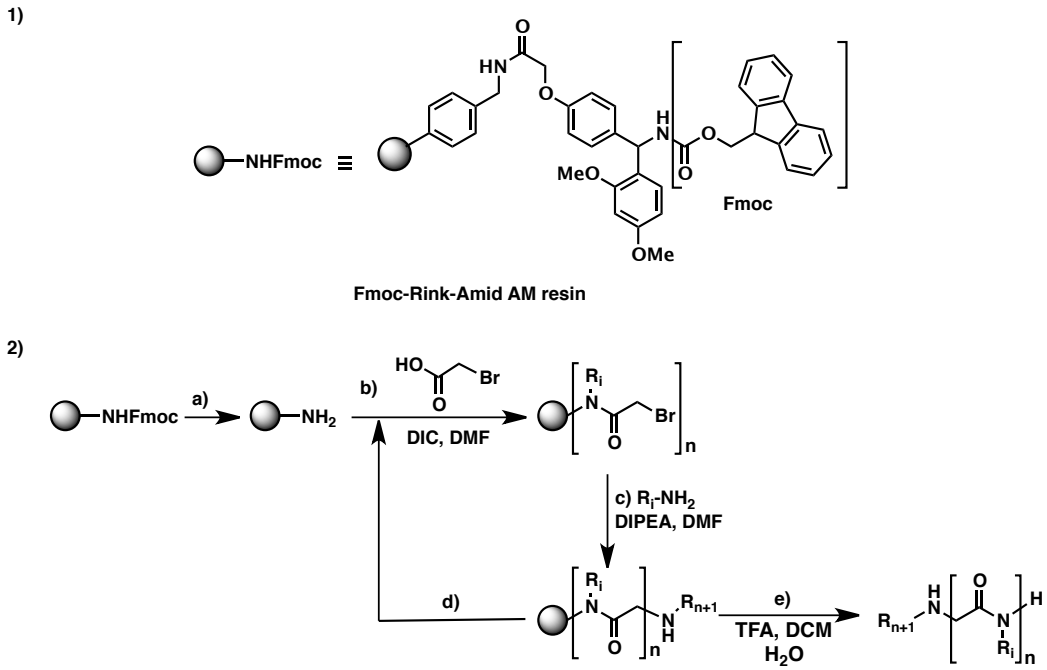


Figure 28. 1) Structure of Rink amide resin with the Fmoc moiety. 2) Sequential synthesis of peptoids. a) Deprotection, b) acylation with bromacetic acid, c) introduction of the diversity by amination, d) repeat b and c to obtain a polypeptoid e) cleavage of the peptoid from the resin.

The general synthetic protocol involved first the deprotection of the resin to generate the free primary amine where the peptoid would grow. After deprotection, peptoids were built using iteratively two successive steps of acylation and displacement. In the acylation step, bromoacetic acid activated by diisopropylcarbodiimide (DIC) reacts with the amine of the previous residue, generating an α-bromo amido derivative. In the displacement step a selected amine substitutes the halide to form the N-substituted glycine residue. When the peptoid reaches the desired length it is cleaved from the resin. As the product is synthesized on solid phase, isolation and characterization of each synthetic intermediate was generally discarded. To have control over reaction progress, different qualitative colorimetric reactions were performed over small aliquots of resin. The 2,4,6-trinitrobenzenesulfonic acid (TNBS) test was used for detecting primary amino groups.[91] Beads containing a primary amine (positive) turned orange-red. Using this test it was possible to recognize if the first amidation over the deprotected resin had been carried out successfully. On

the other hand the chloranil test was developed for a reliable detection of secondary amino groups.[92] It would dye the resin beads of a dark blue to green color in case of a positive, so it was used after each amidation step (Figure 28, b) to confirm the completeness of the reaction.

Synthesized peptoids were purified using semipreparative reverse phase HPLC, using acetonitrile (ACN)-water mixtures containing a small amount of trifluoro acetic acid (TFA) to ensure good resolution of the peaks during the separation. As products were isolated as salts, neutralization was performed before yield determination and characterization were conducted. Products were obtained in purities above 95 % and global yields ranged 25 – 42 %. Peptoids were characterized through Nuclear Magnetic Resonance (NMR) and High Resolution Mass Spectroscopy Electrospray Ionization (HRMS-ESI). NMR spectra were complex due to the existence of several conformers in equilibrium in solution, which were attributed to rotamers of the amide bonds present in the molecule. Those amide bonds block the free rotation of the molecule, generating species that interconvert slowly under the conditions of the NMR-analysis. For this reason the signals of the atoms implied in these equilibriums appear as broad signals or as pairs of signals.

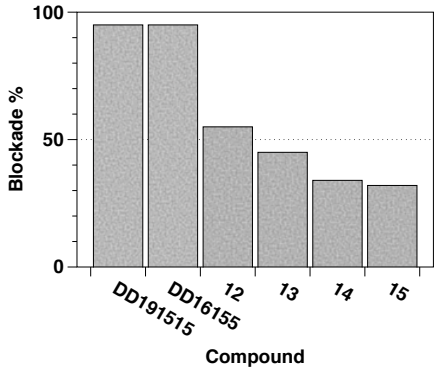


Figure 29. Blocking potency of the initial peptoid hits and derivatives **12-15** assayed at 10 μM.

The electrophysiological technique voltage-clamp was used to measure the blocking potency of these products.[93] This technique will be explained in detail on chapter 4. The determined activities of the four peptoids (Figure 29) provided important SAR information. Exchange of the ethyl by methyl groups in the protonable amine group resulted in some decrease on the TRPV1 blockade activity. Complete removal of the cationic moiety by exchange of the dialkylamino group by a polar hydroxyl end or a non polar propyl chain resulted in further decrease of the activity. On the other hand,

slight modifications on the substitution of the aromatic moieties which imply small changes on the electron density and hydrophobicity of the aromatic ring (**13** vs **12**) had also a slight negative effect on the activity.

2.3 Rigid Scaffold selection.

With the above preliminary results, we decided to turn to the search of a more rigid scaffold. This scaffold should support the desired pharmacophoric features in an optimum 3D arrangement to achieve similar or improved potencies relative to the original hits. In addition it should exhibit some or all of the following properties:

- Commercially available or easily accessible through synthesis.
- At least three tunable substitution positions.
- It would be interesting that the scaffold had previously been used in medicinal chemistry environments, showing low toxicity profiles.
- As the channel itself shows symmetry (i.e. tetrameric structure) and most of the antagonists studied had also some symmetry in the sense that two of the substituents were normally identical, we also hypothesized that certain degree of symmetry could improve the activity and selectivity of the new derivatives.

Some of the candidate scaffolds fulfilling most of the above properties were those of trisubstituted 1,3,5-triazine, purine, thiobarbiturate and *N*-substituted pyrrolidine (Figure 30). From all of them, the 1,3,5-triazine scaffold seemed the most appropriate for the following reasons:

- The syntheses of triazine derivatives are largely described in comparison with the other scaffolds.
- It possesses three tuneable positions that can be substituted in controlled conditions generating symmetric products, if desired.

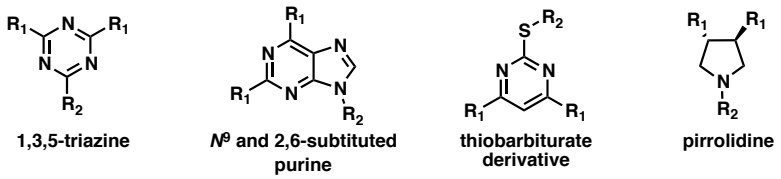


Figure 30. Selected scaffolds to synthesize new derivatives with potential TRPV1 blocking activity.

2.3.1 Triazine properties.

The triazine scaffold is present on different bioactive products (Figure 31), like herbicides (prometryn, simazine), anti-neoplasic agents (altretamine), or respiratory stimulants (almitrine), and is the subject of intense research focused on identifying compounds with activity as antimicrobials,[94] anticancer[95-96] or HIV-1 reverse transcriptase inhibitors.[97]

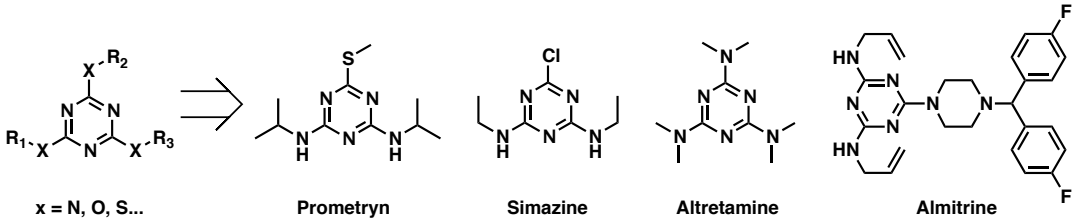


Figure 31. Structure of some relevant triazines with different application.

Although it is a very simple scaffold, it is able to perform different types of interactions with biological receptors[98] due to its highly polarizable conjugated structure (Figure 32).

Different methods have been reported on the preparation of triazines from different starting materials.[99,100] Among them, the preparation of triazine derivatives using 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride, **16**) as starting material seemed to be best option to achieve our desired products.[101-103] 2,4,6-trichloro-1,3,5-triazine is a commercially available cheap reagent (around 40 € per kilogram with 99,5 % purity). It is a dusty white solid with a very strong smell, soluble in most organic solvents. In 2005, approximately 200,000 tones of cyanuric chloride were industrially produced.[104]

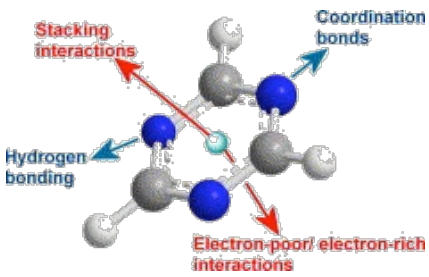


Figure 32: Possible non-covalent bonding properties of the triazine ring. The different interaction properties can be enhanced or diminished based on the substituents.

Commonly, nucleophilic substitution on aromatic systems (S_NAr) does not occur due to the high electronic density of the aromatic ring. However, in the case of cyanuric

chloride the nitrogen atoms on the ring strongly attract the electronic clouds and chlorine atoms in positions 1, 3 and 5 exert a negative inductive effect, draining electronic charge from the ring (Figure 33 and Figure 34).[105]

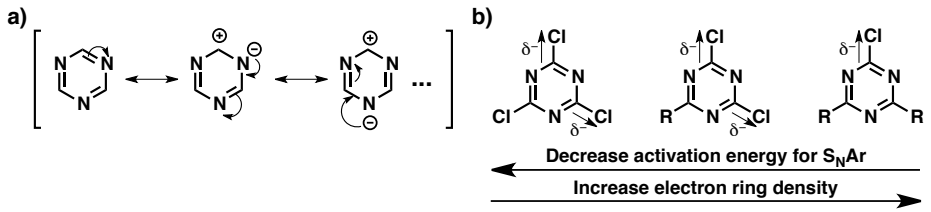


Figure 33. a) Resonant canonical structures of the triazine ring. b) Effects of the exchange of chlorine atoms on the electronic density and activation energy of S_NAr reactions of 2,4,6-trichloro-1,3,5-triazine.

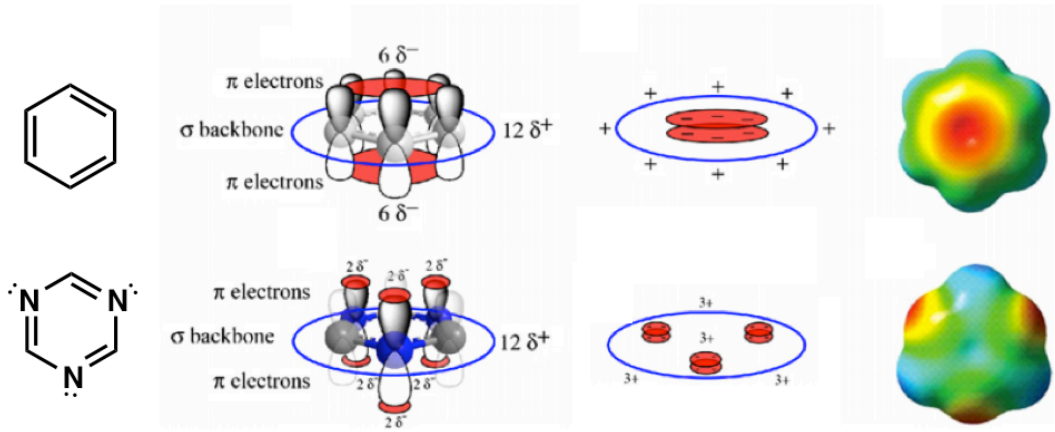


Figure 34. Comparison between the electronic properties of benzene and 1,3,5-triazine. Hydrogen atoms are omitted for clarity. Color code of electrostatic potential surfaces spans from -15 (red) to +15 (blue) kcal mol^{-1} for benzene and from -21 (red) to +21 (blue) kcal mol^{-1} for 1,3,5-triazine.[98]

Because of the above, positions 2, 4 and 6 of cyanuric chloride become suitable for nucleophilic substitution. As chlorines are exchanged for other side chains, the aromatic ring (depending on the substituent introduced) increases its electronic density due to the loss of the negative inductive contribution of chlorine, making more difficult to exchange the next chlorine atom. For that reason the activation energy of the first substitution is lower that the second, and this lower than the third.[106-108]

Until this point all the TRPV1 blockers synthesized in our group had two identical aromatic moieties in their structure, which were not completely equivalent because of their position in the corresponding scaffold. In the case of triazines, introducing two identical subtituents would lead to symmetric compounds that could comply with the assumed pharmacophoric requirements of TRPV1 (Figure 35).

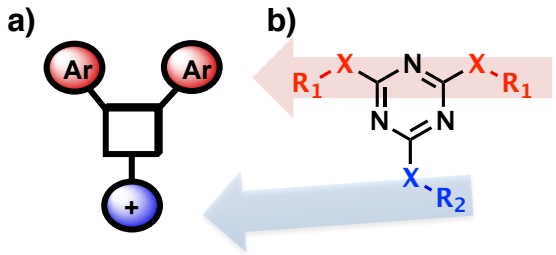


Figure 35. a) Hypothetical three points pharmacophore. Circles represent pharmacophore attributes: In red aromatic residues, in blue cationic moieties. b) Structure of trisubstituted triazines.

To achieve trisubstituted symmetric triazines, two general synthetic routes were considered (Figure 36). *Route A* consists on a double substitution of two chlorines of cyanuric chloride to give a disubstituted intermediate and thereafter a final chloride substitution to yield the desired product. *Route B* involves an initial mono chloride substitution and subsequently a disubstitution over the remaining chlorine atoms.

Route A would generally generate a less reactive intermediate than *route B* because the third chloride substitution of the triazine requires more energy than the previous two chlorine atoms substitution. This would lead to triazines disubstituted with aromatic moieties which will act as chromophoric groups, increasing the molar extinction coefficients of the products.[109] The higher extinction coefficients directly improve the detection by UV-absorption methods, making this route very interesting from a practical point of view.

On the other hand, *route B* allows the introduction of less reactive amines or avoid secondary reactions associated with thermic treatments due the easiness of the first chlorine atom exchange.

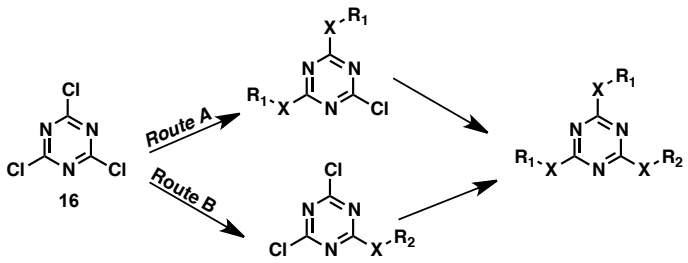


Figure 36. Two routes to achieve trisubstituted triazines based on nucleophilic substitution of **16**.

Finally, since these substitution reactions generate hydrogen chloride, the presence of a base in the reaction media is required to neutralize this acid. This base

could be inorganic carbonate,[101] a non-nucleophylic amine (i.e. triethylamine) or an excess of the nucleophilic amine reagent.[102]

With the above data in mind, the synthesis of triazine **17** (Figure 37) following route *A* was proposed as proof of concept.

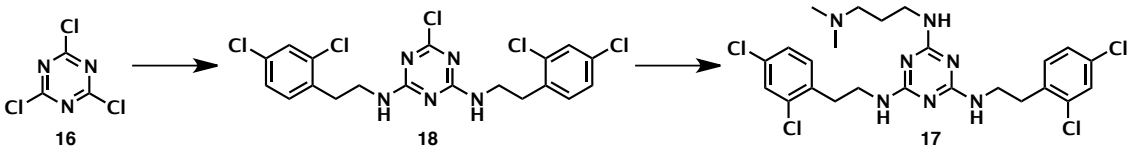


Figure 37. Synthetic route for the preparation of triazine **17**.

Molecule **17** contains two of the side chains that were found to elicit TRPV1 blocking activity in peptoids. On the other hand, the results of preliminary biological evaluation of peptoids **12-15** suggested that the dimethyl and diethylaminopropyl moieties contributed similarly to the activity of the compounds. Because of atom efficiency and because of its lower flexibility, the dimethylamino group was selected over diethylamino group.

2.4 Synthesis of Triazine 17.

2.4.1 Reaction of 16 with 2,4-Dichlorophenethylamine.

Although synthesis of trisubstituted triazines is well documented, there is a large variability on the reaction conditions and purification methods.[95,101,110-113]

The first step in the synthesis of **18** was the disubstitution of two chlorine atoms of cyanuric chloride (**16**) by 2,4-dichlorophenethylamine (**20a**). The reaction was carried out using microwave irradiation to achieve a temperature of 70 ºC, a small excess (2.2 equivalents) of the desired amine, and K_2CO_3 as base.

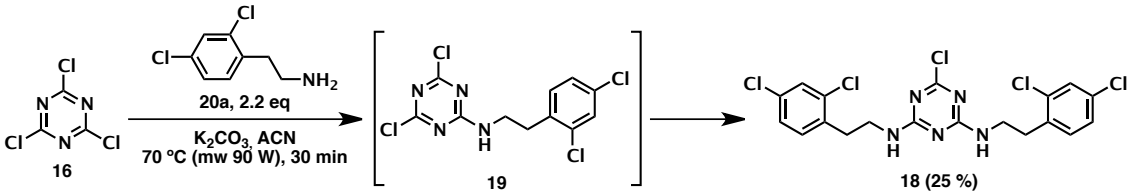


Figure 38. Synthesis of the disusbstituted triazine **18**. Heating was performed by microwave irradiation (mw) with a power of 90 watts.

The reaction was exothermic, generating smoke and vapours as well as a white solid which was insoluble in most organic solvents and which later was identified as a mixture of mono and disubstituted triazine. Small aliquots of the reaction were dissolved in acetonitrile containing 7% (v/v) of TFA and were analysed by HPLC to control the reaction progress. After 30 minutes of reaction, aside from a large peak eluting with the front, which was due to TFA, the HPLC traces showed peaks of the starting reagents (**16** and **20a**), the monosubstituted (**19**) and disubstituted (**18**) triazines, as well as small peaks of impurities which were not identified (Figure 39).

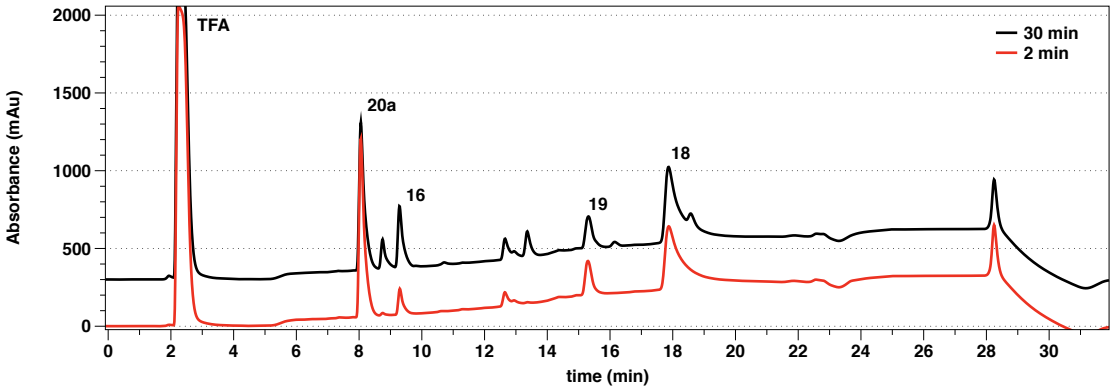


Figure 39. Reverse phase HPLC traces of aliquots of the crude reaction mixture at 2 (red) and 30 (black with an ordinate offset of 300 mAbs units) minutes of reaction, using UV-detection at 220 nm. Assignment based on the m/z value and the isotopic profile of the $M+H^+$ peak detected by HRMS-ESI.

Since no further progress was observed, the reaction was stopped. The fact that the crude reaction mixture was insoluble was a great handicap to apply purification techniques as crystallization or chromatography. However, washing the solid with THF allowed to remove almost selectively all the impurities present, yielding a solid product which was essentially the desired disubstituted triazine **18** (Figure 40).

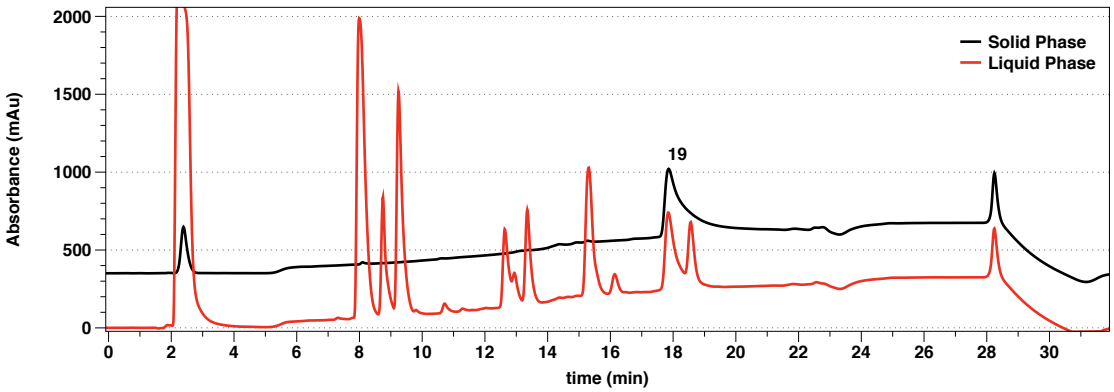


Figure 40. Comparison of the reverse phase HPLC traces of the washing organic phase and the remaining solid material obtained after the workup.

This product was used without further purification for the next reaction, despite the yield of this unoptimized step was only 25 %.

2.4.2 Reaction of 18 with 3-Dimethylamino-1-propylamine.

The solid obtained in the previous step was suspended in *N,N*-dimethylformamide (DMF) and, following the conditions described by Diaz-Ortiz et al.,[102] an excess of 3-dimethylamino-1-propylamine (4 eq) was added. The mixture was subjected to a single microwave cycle (90 W, 130 ºC) in a sealed flask for 10 min.

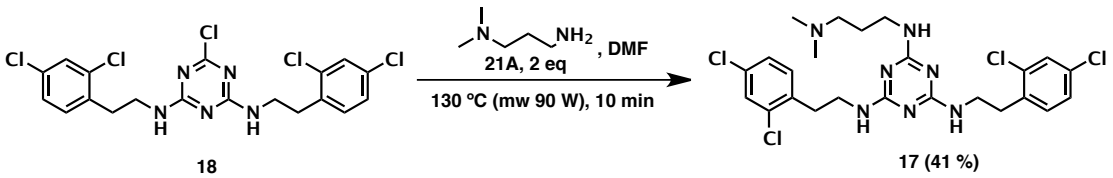


Figure 41. Reaction scheme to obtain **17** starting from **18**. Reaction was performed in a sealed flask under microwave irraditation.

After this cycle the suspension was totally dissolved. The HPLC analysis showed a total conversion of the starting material to form of a single major product (Figure 42), which by HRMS-ESI detection was attributed to the desired triazine **17**. The crude was purified by semipreparative HPLC to yield pure **17** in 41% yield.

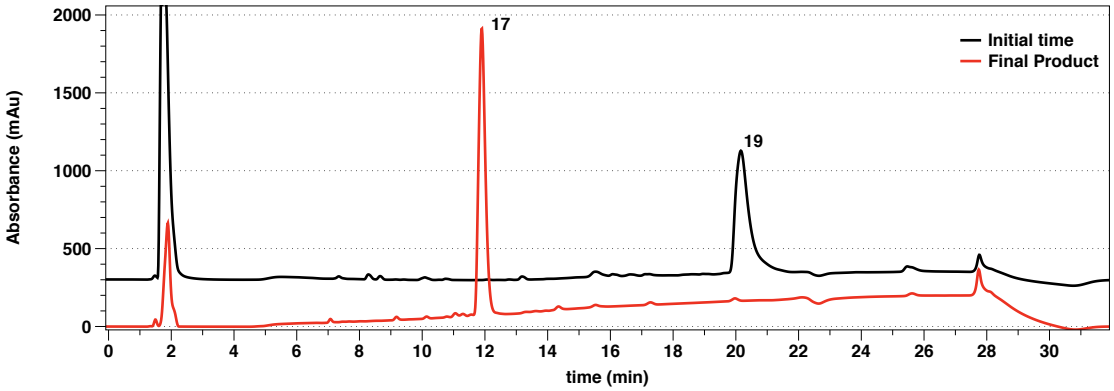


Figure 42. Reverse phase HPLC traces of aliquots of the reaction crude at starting and end time.

Triazine **17** showed a promising activity as antagonist of TRPV1**:** At 10 μM, it blocked 79 % of the capsaicine evoked current. Furthermore, from the dose-response curve, an IC_{50} of 1.2 μM was estimated. This activity was the highest obtained for any cyclic derivative up to that point, which encouraged us to build up a triazine library with the aim of identifying new TRPV1 antagonists.

2.5 Optimization of a microwave based method for the synthesis of 2,4,6-trisubstituted-1,3,5-triazines.

In order improve the yields and purity of the synthesized triazine, relevant reaction parameters had to be identified and analyzed. The following parameters were considered: heating mode, solvent, reaction temperature, microwave power, reagent and equivalents.

2.5.1 Heating mode.

Traditional heating, based on conduction/convection, heats directionally from the outside to the inside of the reaction container due to the thermal gradient that exists between the reaction vessel and the heat source. Reported synthetic methodologies for 2,4,6-trisubstituted-1,3,5-triazines based on this heating mode might require several days of reaction at high temperature.[111,114]

On the other hand, microwave ovens heat directly the bulk of the reaction due to an unfocused increase of the rotational energy of molecules. Our group had a large experience using microwave irradiation to activate nucleophilic substitutions in the synthesis of peptoids and peptoid analogues.[74,115,116] Routinely, reactions are carried out in a CEM Discover microwave oven, with variable power controlled by a computer, or, if the reaction is robust and no reflux is required, with a conventional kitchen microwave oven. Microwave heating, presents the following benefits over conduction/convection heating.[117,118]

1) Microwaves directly heat the core of the reaction (Figure 43). This implies faster and efficient heating with little heat diffusion through the reaction vessel.

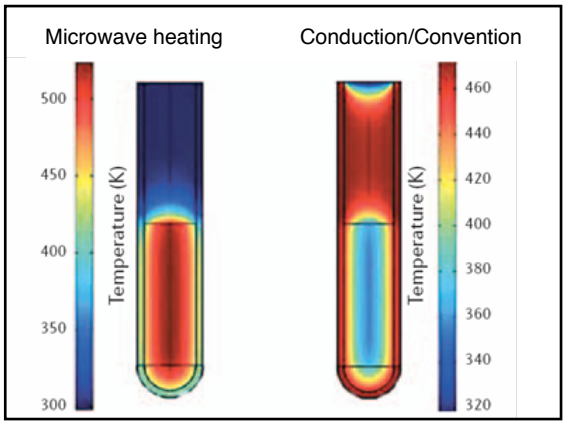


Figure 43. Comparison between temperature gradient in convection/conduction and microwave heating of a tube reactor.

2) “Microwave effects”, that occur only under microwave irradiation, like (i) selective heating of specific reaction components, (ii) rapid heating rates and temperature gradients, (iii) the elimination of wall effects, and (iv) the superheating of solvents.
3) In most cases the reaction times are reduced drastically, the crude reaction mixtures have less side products and higher yields are obtained.
4) The use of sealed tubes provides a method to overheat solvents above the boiling point. This contributes to the kinetics of the reaction, since higher activation energies can be achieved.
5) Using a computer connected to the microwave oven allows to monitor the pressure and temperature on real time, aiding to make reproducible experiments.
6) It can be easily automated and adapted for parallel or sequential synthesis.
Thus, microwave activation was the chosen method to activate the reactions.

2.5.2 Solvent.

In our case, the study of the solvent effect was restricted to the use of DMF, ACN or tetrahydrofurane (THF), as they are the most frequently used solvents in this type of reactions.[96,102,103,112,119]

2.5.3 Reaction temperature and microwave power.

Typical reaction temperatures required for substitution of each chlorine atom of **16** are:[102,111,113]

1st substitution: low to ambient temperatures.

2nd substitution: moderate temperatures (< 80 °C).

3rd substitution: high temperatures (up to ~180 °C).

The use of microwave irradiation implies that another variable related with temperature comes into play: the irradiation power (in W). Microwaves can activate directly the reaction without increasing the temperature if solvent does not interact with the microwave radiation, or, if it does, they can increase the temperature of the solvent to activate the reaction. Thus, different values of temperature/power were tested.

2.5.4 Reagents and equivalents.

As previously stated, substitution reactions on **16** release HCl, therefore the use of a base or an excess of the nucleophilic amine were explored.

2.5.5 Optimization of the general synthetic procedure.

Nucleophilic substitution of cyanuric acid (**16)** with amine **20a** to produce the disubstituted triazine **18** was the first reaction studied. HPLC analysis of the crudes was used to determine the conversions. Table 1 summarizes the different conditions assayed and the results obtained.

Under solvent-free conditions (test 1) a complex crude was formed and although the peak of starting material **16** disappeared completely, **18** was formed as a minor product. In the case of assays using ACN as solvent, the conversion to **18** was not complete (test 2), even using a large excess of amine **20a** (test 3). Using DMF as solvent, **16** disappeared but no detectable peak of **18** was observed (test 5). It seems that under these conditions, **16** reacts with DMF to form the so called Gold's reagent[120] (Figure 44), consuming the starting material but not yielding the desired product.

Table 1. Summary of the different experimental conditions assayed for the synthesis of **18**. The relative peak areas (%) of compounds **16** and **18**, determined from the HPLC-UV (λ = 220 nm) profiles of the crudes at the specified time, were used to determine reaction conversions.

Test	Eq 20a	Base	Temp (ºC)	t (min)	P (W)	Solvent	% area 16	% area 18
1	2	$NaHCO_3$	130	10	90	---	0	33
2	2.2	K_2CO_3	70	10	90	ACN	20	66
3	8	---	70	10	90	ACN	30	28
4	3 + 1.5	---	70	20	90	THF	15	70
5	4	---	70	20	90	DMF	0	0
6	---	---	70	20	90	DMF	0	0
7	4	---	70	20	90	THF	0	98

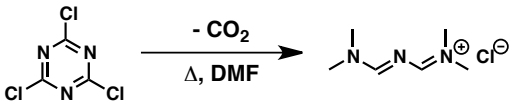


Figure 44. Gold's reagent formation from **16** and DMF at high temperature.

On the contrary, using THF as solvent, at 70 ºC in a sealed flask, and 4 equivalents of amine **20a** almost quantitative conversions were achieved (Tests 4 and 7), therefore these conditions were chosen as an efficient method to produce intermediate **18**. It is worth noting that although microwave power was initially set up at 90 W, the irradiation power was automatically reduced by the microwave oven as the reaction progressed, to maintain the temperature at 70 ºC. Thus, the mean value of

power irradiation during this process was around 30 W, and the higher value was only relevant for the initial heating of the reaction.

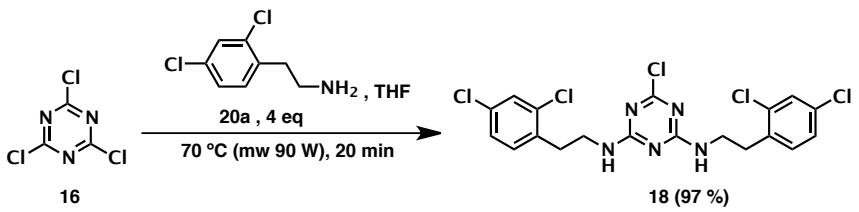


Figure 45. Synthetic scheme of **18** from **16** under optimized conditions.

Concerning the purification of triazine **18**, the product is a white dusty solid, odourless, not hygroscopic and insoluble in most solvents. Small aliquots (1 mg) of **18** were employed to test its solubility at room temperature, checking the results by naked eye. **18** appeared as insoluble at 25 ºC in MeOH, ACN, THF, triethylamine (TEA), dimethylsulfoxide (DMSO), ethyl acetate (EtOAc), H_2O, acetic acid (AcOH), and acetone, partially soluble in DMF, and soluble in ACN with 7% (v/v) TFA. At 60 ºC, **18** was soluble in AcOH, THF with 10 % (v/v) AcOH, DMF, acetone, and ACN with 7% (v/v) TFA. Attempts of purification of **18** by crystallization using different solvent mixtures were unsuccessful. However, its differential solubility relative to the precursor reagents and by-products formed during the reaction was used to set up a purification protocol.

Thus, after the established reaction time, the reaction crude mixture was suspended in a large volume of water, and heated gently under vacuum to evaporate the THF. Then, the suspension was allowed to cool, the solid was filtered and repeatedly washed with cold water, and finally with cold EtOH. The addition of water to the crude suspension and removal of the THF by evaporation ensured the precipitation of **18** and rests of unreacted monosubstituted intermediate **19**, while the excess of amine **20a** would remain in the aqueous phase as chlorhydrate. The final wash of the precipitate with EtOH probed to be effective to selectively remove **19**. Therefore, with this method, pure **18** (>95 % by HPLC) could be afforded without using more complicated or expensive separation protocols.

Substitution of the third chlorine atom of **18** implied a higher activation energy. Diaz-Ortiz *et al.*[102] reported a method to substitute the last chlorine atom from a disubstituted triazine employing a microwave oven to heat the reaction. Using similar conditions (Table 2), which required carrying the reaction at high temperature (110 ºC) in a sealed flask, the last chlorine atom was substituted quantitatively with no side

reactions. As above, taking advantage of the solubility of the chlorhydrate of amine **21A** in aqueous media, the final product **17** was obtained essentially free from impurities by partitioning the crude reaction mixture in EtOAc and saturated $NaHCO_3$ solution.

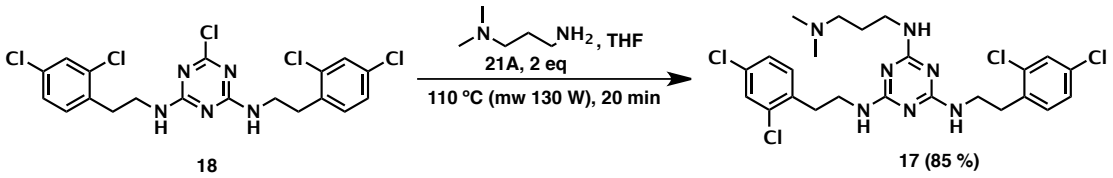


Figure 46. Synthetic scheme to prepare **17** from **18**.

Table 2. Reaction conditions for third chlorine substitution.

Test	Eq 21A	Temp (ºC)	t (min)	P (W)	Solvent	% area 18	% area 17
1	2	110	20	130	THF	< 1	92

Although the purity of triazine **17** obtained by this method was >90 % by HPLC and enough for most biological testing, the compound was further purified by preparative reverse phase HPLC when required. Global yield achieved was 85 % relative to the limiting reagent **16**.

In summary this synthetic protocol had the following advantages:

a) Reactions that conventionally would take days, were completed in less than one hour.
b) Faster purifications without the use of silica or preparative HPLC were used, implying better yields.
c) Lower consumption of solvents and resources.

We envisioned that this protocol could be applied to the preparation of a wider range of trisubstituted triazines, thus the synthesis of a small library of compounds was planned next.

2.6 Synthesis of a focused chemical library of 2,4,6-trisubstituted-1,3,5-triazines.

Based on the preliminary results of activity as TRPV1 blocker obtained for triazine **17**, we decided to design a small library of compounds that include structural

variations in the side-chains of the triazine, in order to obtain SAR information and eventually develop a QSAR (Quantitative Structure-Activity Relationships) model.

Since at the time this thesis was conducted little was known about the potential binding site on TRPV1 for these uncompetitive inhibitors, the selection of side-chains was carried out trying to include a structurally diverse set of amines, which were the formal source of diversity, from commercial catalogs. These amine reagents *a priory* would have to include aromatic moieties or substituted alkyl moieties to introduce the corresponding groups in the substituted triazines, and conform to the postulated TRPV1 pharmacophore (Figure 35, pp. 51). In addition, since the synthesis of the new compounds was carried out at the same time that the biological assays were performed (Chapter 4), the preliminary results obtained also conditioned the triazines that were finally prepared.

Therefore, the following amines were considered as diversity sources (Figure 47):

- For R_1:

a) Phenethyl amines (**20a-h**): These were analogues of the susbtituents found on the initial peptoid hits and the first active triazine derivative **17**, and would allow to check how the different substitution site on the aromatic ring or the different electronic properties of the substituents affect the activity of the triazine.

b) Benzylic amines (**20i-q**): Benzylic amines have a shorter alkyl chain and are therefore less flexible than the previous ones. In addition, amines **20p** and **20q** were chosen not only to check the effect of the carboxylate as substituent of the aromatic ring but also to introduce a reactive site for further modification.

c) Amines including more complex aromatic systems (**20r-t**): These were selected to check the effects of larger groups which could interact with electron deficient regions of the receptor, as well as with hydrophobic and aromatic residues, or which could reveal steric limitations on the binding site. Amine **20t** was the one with the bulkiest side chain introduced into the triazine scaffold.

d) Finally, amine **20u** was selected as a probe to test the contribution of the aromatic moiety in R_1 to the activity of the triazine derivatives.

- For R_2:

a) Linear alkylic amines containing a cationic group (**21A-G**): Selected amines exhibit different lengths between the reactive amine and the cationic center. Reagents **21F** and **21G** also included the bioisosteric replacement of the reactive amine (as in **21A**) or a methylene group (as in **21C**) by an alcohol or ether group, which would led to electronegative oxygen containing triazine derivatives.

b) Amines containing bulky cationic moieties (**21H-M**): Piperazine, piperidine and pyrrolidine containing amines were selected to determine their effect on activity.

c) Amines containing neutral side-chains (**21N-Q**): Selected to assess the requirements of a cationic moiety for activity.

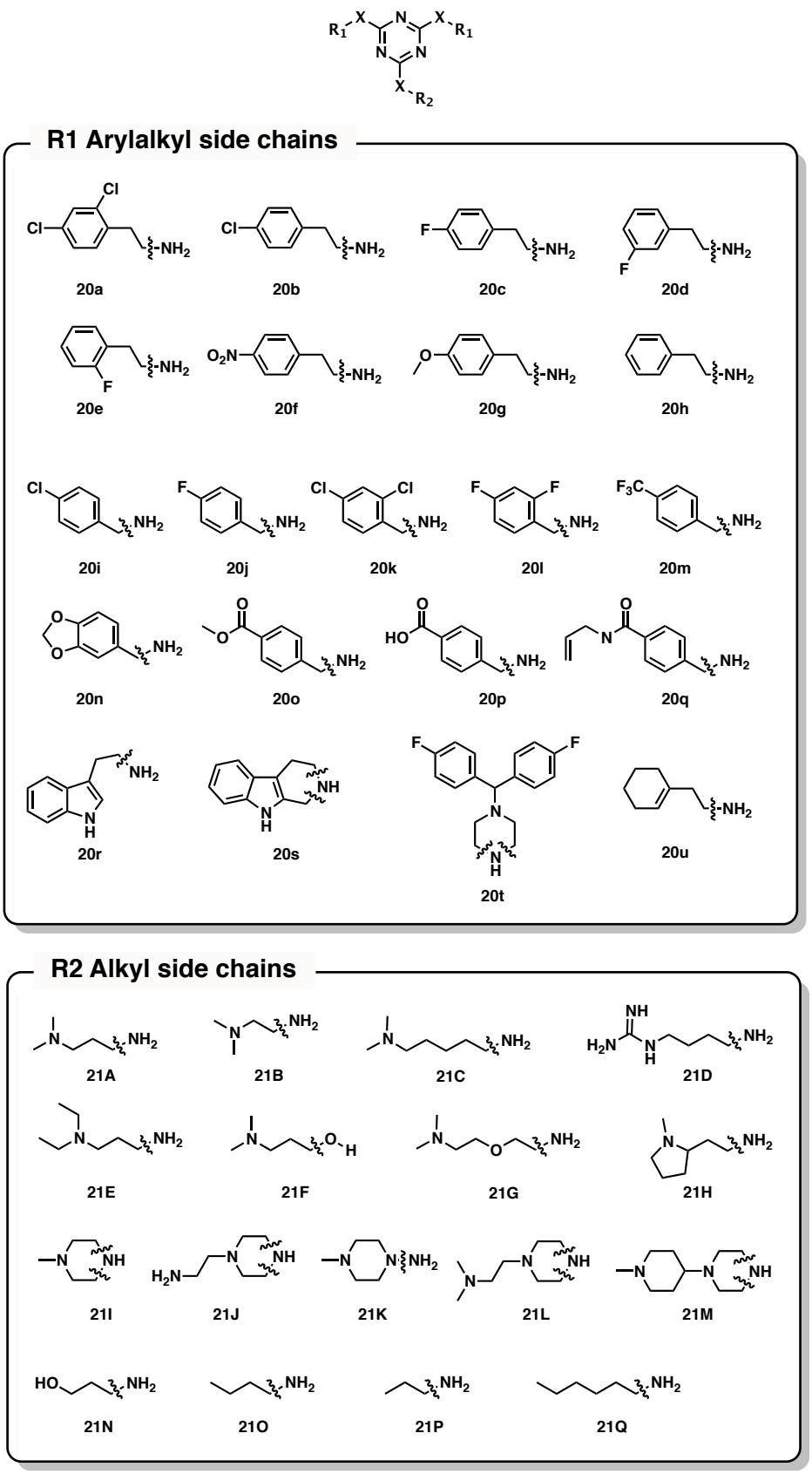
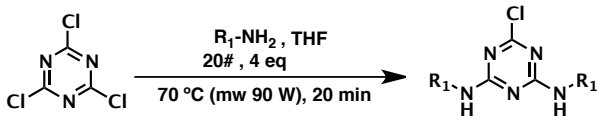


Figure 47. General structure of the triazine derivatives and formal diversity sources considered. Most of these were commercially available amines, however **21D** was not directly available and had to be introduced following a specific strategy (see below).

2.6.1 Synthesis of symmetric 2-chloro-4,6-disubstituted-1,3,5-triazines.

The optimized procedure previously described was employed to synthesize the collection of disubstituted derivatives **18**, **22-39** (Table 3).

Table 3: Synthetic scheme of symmetric disubstituted triazines starting from cyanuric chloride (**16**) and a collection of amines (**20#**). The table shows the yields and max amount of each synthesized product per batch.



16 **18, 22-39**

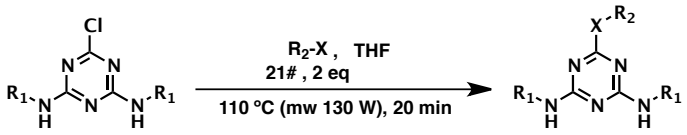
Product	R_1	Yield	Max amount synthesized (mg)	Product	R_1	Yield	Max amount synthesized (mg)
18	**20a**	97	262	**31**	**20k**	95	263
22	**20b**	94	1190	**32**	**20l**	87	206
23	**20c**	83	2270	**33**	**20m**	89	170
24	**20d**	95	205	**34**	**20n**	87	390
25	**20e**	95	210	**35**	**20o**	42	96
26	**20f**	93	250	**36**	**20r**	42	200
27	**20g**	93	210	**37**	**20s**	81	200
28	**20h**	89	170	**38**	**20t**	54	205
29	**20i**	92	200	**39**	**20u**	87	340
30	**20j**	60	110				

The products were usually solids, stable to the air and insoluble in most solvents. Indole derivatives (**36** and **37**) were found to be sensible to air exposure in aqueous acid media, forming laminar black insoluble solids probably due to polymerization.[121] Yields ranged from 42 to 97 % depending on the amine used. Thus, the generality and efficiency of the protocol previously set up for the preparation of disubstituted triazines analogues of **18** was confirmed. All the disubstituted triazines were characterized by ^{1}H-NMR spectroscopy and HRMS-ESI.

2.6.2 Synthesis of the symmetric 2,4,6-trisubstituted-1,3,5-triazines.

Using the optimized methodology detailed for the synthesis of triazine **17**, the trisubstituted triazines **40-74** were synthesized (Table 4). However, triazines **47**, **48**, **50**, **69** and **70** required some adaptations of the synthetic methodology which are described below.

Table 4: Synthetic scheme of symmetric trisubstituted triazines starting from corresponding disubstituted ones (**18**, **22-39**) and a collection of amines (**21#**). The table shows the yields and amounts of each synthesized product per batch.



18, 22-39 17, 40-74

Product	R_1	R_2	Yield	Max amount synthesized (mg)	Product	R_1	R_2	Yield	Max amount synthesized (mg)
17	**20a**	**21A**	88	100	**57**	**20d**	**21A**	53	133
40	**20b**	**21A**	71	64	**58**	**20e**	**21A**	57	143
41	**20b**	**21B**	71	102	**59**	**20f**	**21A**	80	97
42	**20b**	**21H**	60	70	**60**	**20g**	**21A**	57	87
43	**20b**	**21I**	36	52	**61**	**20h**	**21A**	74	105
44	**20b**	**21J**	64	116	**62**	**20i**	**21A**	68	110
45	**20b**	**21K**	55	81	**63**	**20j**	**21A**	72	105
46	**20c**	**21A**	73	456	**64**	**20k**	**21A**	20	38
47	**20c**	**21C**	23	48	**65**	**20l**	**21A**	40	52
48	**20c**	**21D**	23	60	**66**	**20m**	**21A**	96	220
49	**20c**	**21E**	95	129	**67**	**20n**	**21A**	94	226
50	**20c**	**21F**	38	90	**68**	**20o**	**21A**	42	97
51	**20c**	**21G**	23	50	**69**	**20p**	**21A**	61	160
52	**20c**	**21L**	73	192	**70**	**20p**	**21O**	25	27
53	**20c**	**21M**	95	250	**71**	**20r**	**21A**	34	20
54	**20c**	**21N**	91	100	**72**	**20s**	**21A**	63	80
55	**20c**	**21P**	53	84	**73**	**20t**	**21A**	80	120
56	**20c**	**21Q**	73	85	**74**	**20u**	**21A**	95	220

Trisubstituted triazines were usually transparent and stable oil-gel products. Their solubility was generally better than that of the corresponding disubstituted precursors, being soluble in most polar or mid polar solvents (AcOEt, dichloromethane (DCM), acetone, EtOH). Yields were in most cases >50 % although in a few cases they only reached around 20%. All compounds were characterized by ^{1}H- and ^{13}C-NMR spectroscopies, as well as high resolution mass spectrometry (HRMS- ESI), however the routine ^{1}H-NMR spectra were in general too complex as to provide much structural information due to the existence of several conformational species in equilibrium. This will be described in detail in the next chapter.

2.6.3 Synthesis of triazines with specific protocols.

*2.6.3.1 Synthesis of triazine **47**.*

Synthesis of product **47** was attempted using the general procedure but different side-products were formed. The crude reaction mixture was analyzed by UPLC-MS, which allowed to identify two products with M+H of m/z= 399.2109 (**75**) and m/z= 439.2422 (**76**) (Figure 48).

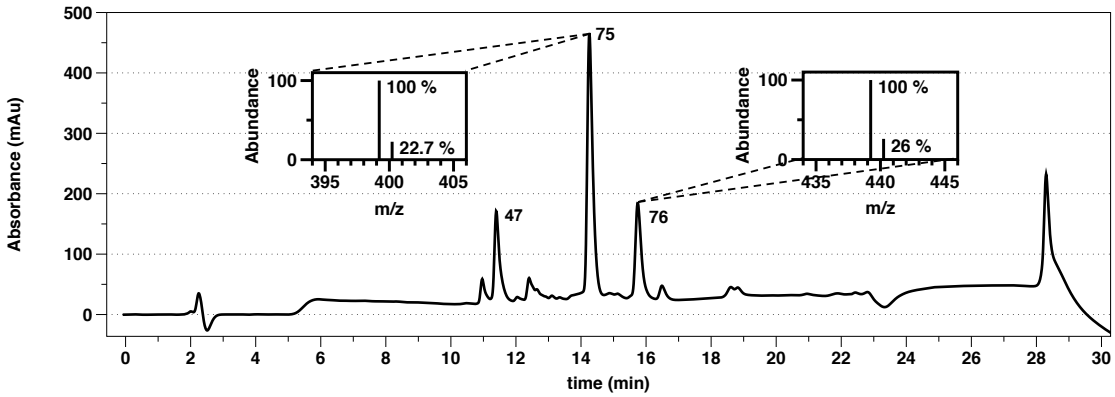


Figure 48: Crude reaction mixture belonging to the synthesis of product **47** showing two mass spectra insets of hypothesized products **75** and **76**.

The M+H ion of these products was compatible with: a) a product where the lineal amine had been cleaved from the triazine and replaced by the dimethylamino group (**75**), and b) the elimination of the dimethylamino group, leaving a piperidine ring bound to the triazine ring (**76**). It was speculated that formation of these products could be promoted by the acid released from the attack of the side chain of amine **21C** on triazine **47** (Figure 49).

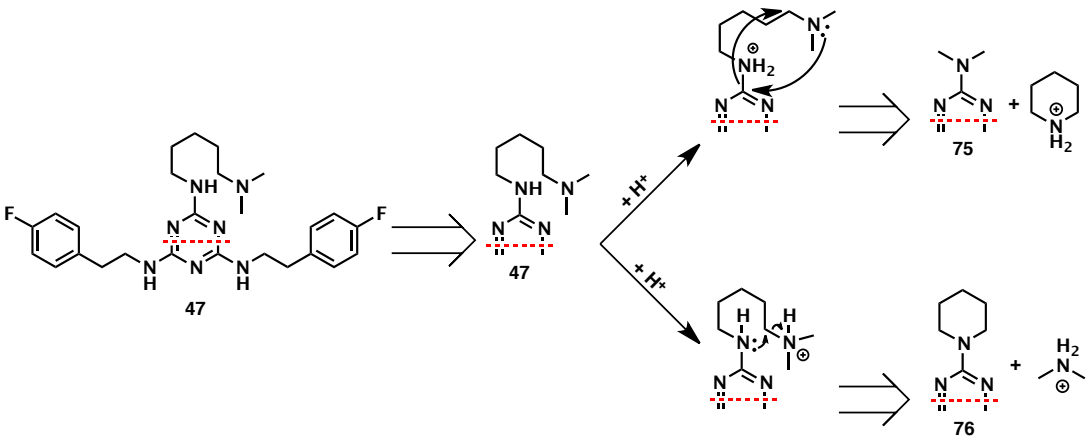


Figure 49: Hypothesized elimination mechanisms that can occur during the synthesis of **47**.

As the interest product was generated only as a minor product, the reaction was assayed at lower temperatures and longer reaction times. The best conditions were those reflected in Figure 50 and despite the low yields, the final product could be obtained with the required purity and quantity.

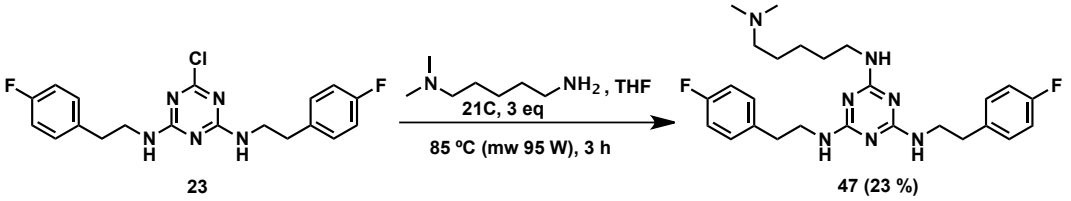


Figure 50. Synthetic scheme of **47** under the best experimental conditons.

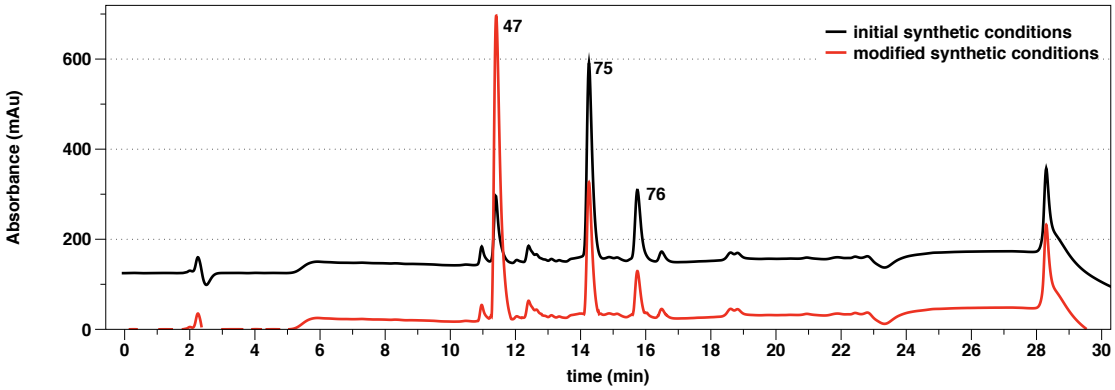


Figure 51. Comparison of crude reaction mixtures from the synthesis of triazine **47** using the general synthetic conditions and the conditions shown in Figure 50. Assignment of the peaks to products **47**, **75** and **76** was based on the corresponding UPLC-MS spectra.

In this context triazine **56** was synthesized with the general procedure as a model to check if issues in the reaction to form **47** were caused because of the length of the alkyl chain. Synthesis of **56** proceeded without issues, generating the desired product without any important side products.

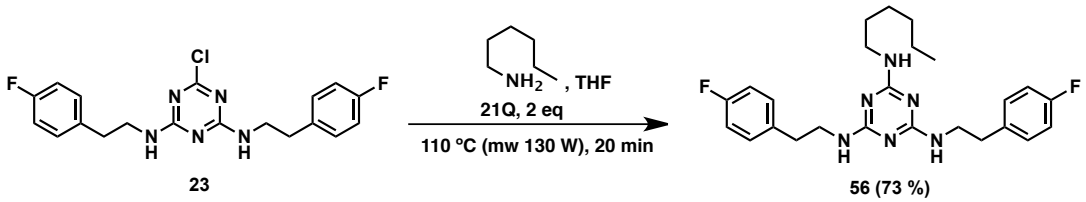


Figure 52. Reaction model to check the anomalous reactivity of amine **21C** vs amine **21Q**.

This pointed in the direction that the trigger of the side reactions observed for **47** was the dimethylamino on side chain **21C**, which is at a proper distance for the intramolecular attack of the triazine-bound nitrogen. Other triazines with shorter alkyl

chains would require to pass through a more strained transition state (Figure 53), not generating this side reaction.

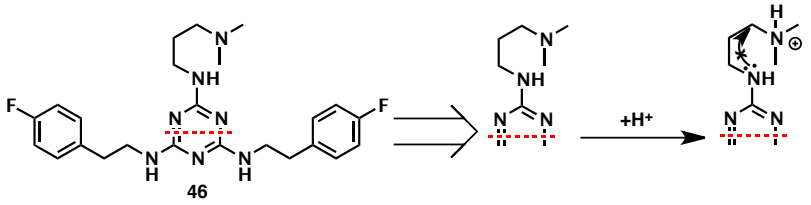


Figure 53. Structure of product **46** showing the impossibility to generate side products as product **47** due the short length of the protonable side chain.

*2.6.3.2 Synthesis of triazine **48.***

The guanidine group present in **21D** could be formally obtained by guanilidation of the Fmoc protected amine of precursor **77**. However, attempts to apply the general synthetic route to prepare triazine **78** from amine **77** and the disubstituted triazine **23** were unsuccessful, yielding complex mixtures where the desired product could not be detected.

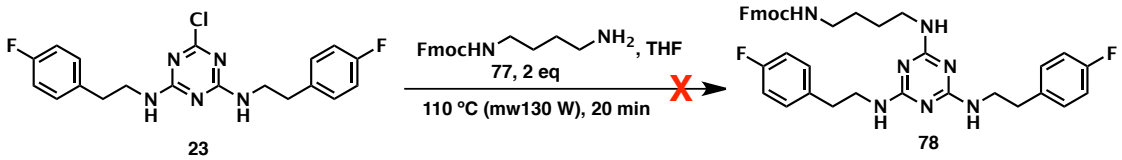


Figure 54. Conditions assayed for the preparation of the trisubstituted triazine **78**.

Therefore, a different route had to be devised for the synthesis of triazine **48**. Thus, in this case an inverted sequence was envisioned where the triazine monosubstitution with **77** was performed first, followed by the disubstitution with the aryl containing amine **20c** (Figure 55). The guanilidation protocol was based on the work published by Feichtinger et al.[122] that used the *N,N′*-di-Boc-*N′′*-triflylguanidine (**80)** as guanilidation reagent. The monosubstitution of **16** with amine **77** to provide triazine **79** in the first step of the synthesis required harder conditions than those previously used to generate other monosubstituted triazines. Product **79** was not isolated nor characterized and the next synthetic step was carried out directly over the crude reaction mixture, by addition of **20c** and reaction under the conditions shown in Figure 55. Once product **78** was obtained, a three-step sequence was employed to yield **48**. First, the cleavage with piperidine of the Fmoc protecting group of **78** and the guanidilation with **80** were performed at room temperature. Using this method the

guanidine introduced is protected with two Boc groups. Thus, further Boc deprotection was required to yield a crude reaction mixture where **48** was the major product. After purification by preparative HPLC, **48** was isolated with an acceptable yield after 5 synthetic steps (23%).

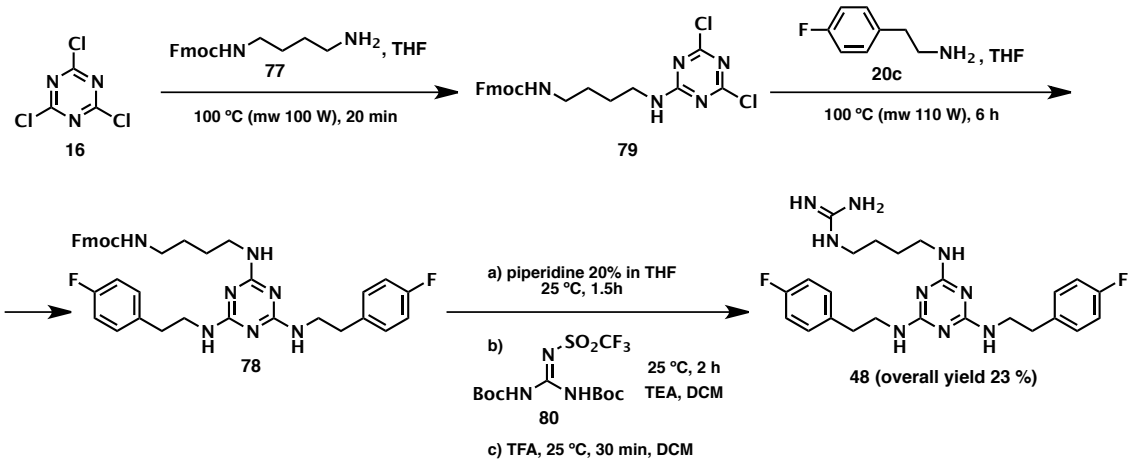


Figure 55. Synthetic strategy for the preparation of product **48** from **16**.

*2.6.3.3 Synthesis of triazine **50.***

Compound **50** was the only product synthesized with an oxygen atom directly linked to the triazine ring. Substitution on triazine **23** was carried out directly using the corresponding alcohol **21F**. Reaction conditions were extracted from the work published by Dudley et al.[108]. The procedure consisted on heating a mixture of **23** suspended in **21F**, which also act as solvent, at 120 ºC for 12 h, in the presence of anhydrous Na_2CO_3. Although the yield of isolated product was relatively low (38%), it was enough to characterize its structure and to evaluate its biological activity.

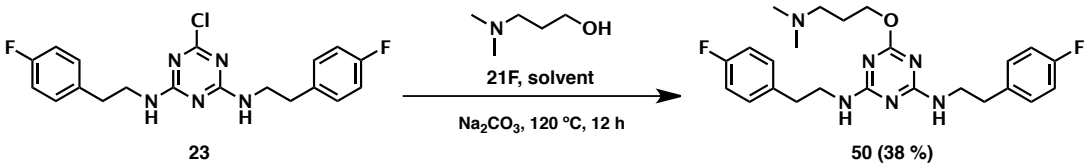


Figure 56. Synthesis of **50** from **23**. Aminoalcohol **21F** was used as solvent and reagent.

*2.6.3.4 Synthesis of triazines **69** and **70.***

Acid derivatives **69** and **70** were obtained from their corresponding methyl ester precursors by means of a standard hydrolytic protocol using sodium hydroxide 0.1 N in 3:2 methanol-water as solvent. Product **69** was obtained directly by treating **68** with the

mentioned hydrolysis mixture for 4 h at 40 ºC, which rendered a crude that after purification yielded product **69** with 61 % yield.

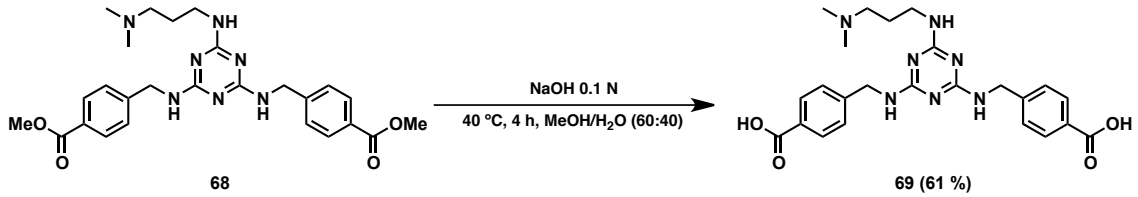


Figure 57. Hydrolysis scheme to obtain product **69** from ester **68**.

On the other hand, product **70** was synthesized starting from the disubstituted **35**, using the general method for the preparation of trisubstituted triazines to add amine **21O**. However, due to difficulties during the purification of the trisubstituted intermediate using the standard partition methodology, the hydrolysis was applied directly over the reaction crude mixture. After the hydrolytic treatment over the crude reaction mixture containing triazine **81**, the crude was purified by semi-preparative HPLC to render product **70** in 27 % yield.

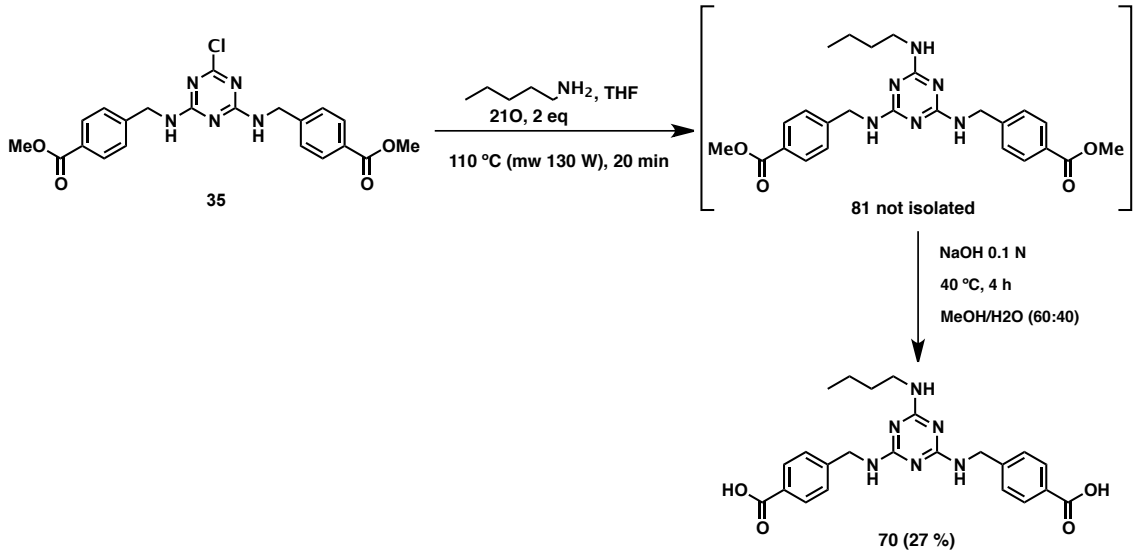


Figure 58. Synthesis of product **70** starting from the disubstituted triazine **35**. The hydrolysis protocol was performed over the crude reaction mixture containing the trisubstituted triazine **81**.

*2.6.3.5 Synthesis of asymmetric trisubstituted triazines **82** and **83.***

The main idea behind the synthesis of asymmetric trisubstituted triazines was to have active TRPV1 blockers which include a center for further derivatization. This would allow linking the triazine to different molecules to add other desired properties,

such as modification of the logP, biochemical trafficking and signalling, or the capacity to interact with other receptors.

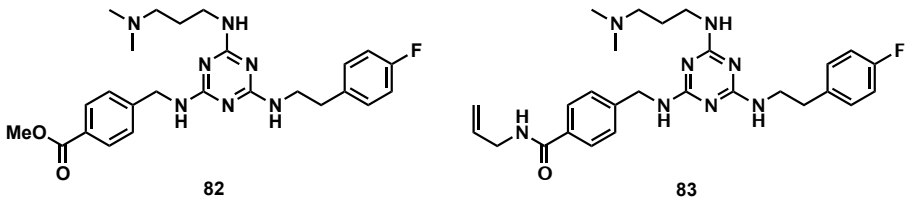


Figure 59. Structure of asymmetric triazines **82** and **83**.

Compounds **82** and **83** include a methyl ester or an allyl amide group masking a carboxylate in a side chain, which could be used for the above mentioned derivatizations. In addition, **82** and **83** bear two additional side chains (**21A** and **20c**) which were also found in the most active triazines (Chapter 4) and were expected to provide activity against TRPV1.

The initial point for the synthesis of triazines **82** and **83** was the asymmetric disubstituted triazine **84**. **84** was synthesized in two steps, starting with the monosubstitution of cyanuric chloride (**16**) with amine **20c** to render intermediate **85**, followed by reaction with amine **20o** (Figure 60).

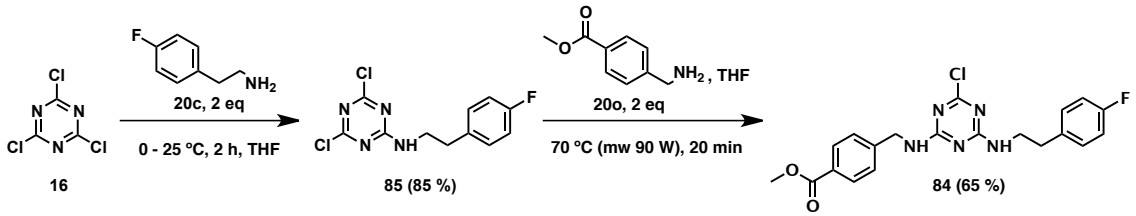


Figure 60. Reaction scheme to synthesize **84** from **16** in two steps.

Monosubstitution of cyanuric chloride (**16**) was carried out by dropwise addition of a solution of amine **20c** at 0 ºC, to control the reaction temperature and to avoid disubstitution reactions. Once all amine was added, the mixture was allowed to reach room temperature and to react for 2 more hours. The crude reaction mixture showed **85** as major product and a small amount of **23**. **85** was quite insoluble, like most disubstituted triazines, and thus a similar work-up procedure was employed. The main difference laid in the final wash with ethanol which was changed to methanol, since solubility assays demonstrated that methanol dissolves better the disubstituted derivative, while ethanol dissolves better the monosubstituted triazine **85**. With this procedure, **85** was obtained in good yields (85%) and purity. Then, monosubstituted **85**

was converted into **84** in reasonable yield (65%), by reaction with amine **20o** under the conditions of the general method for triazine disubstitution. Finally, **82** was obtained from **84** in 90 % yield by applying the standard methodology (Figure 61).

Amidation of methyl ester **82** to furnish **83** was carried out using the desired amine as solvent and microwave irradiation. The yield of this step was very low (17 %), probably due to polymerization of both the desired product and allylamine, but it generated the required quantity of product to perform the structural characterization and the corresponding biological assays, therefore no further optimization of the reaction was attempted.

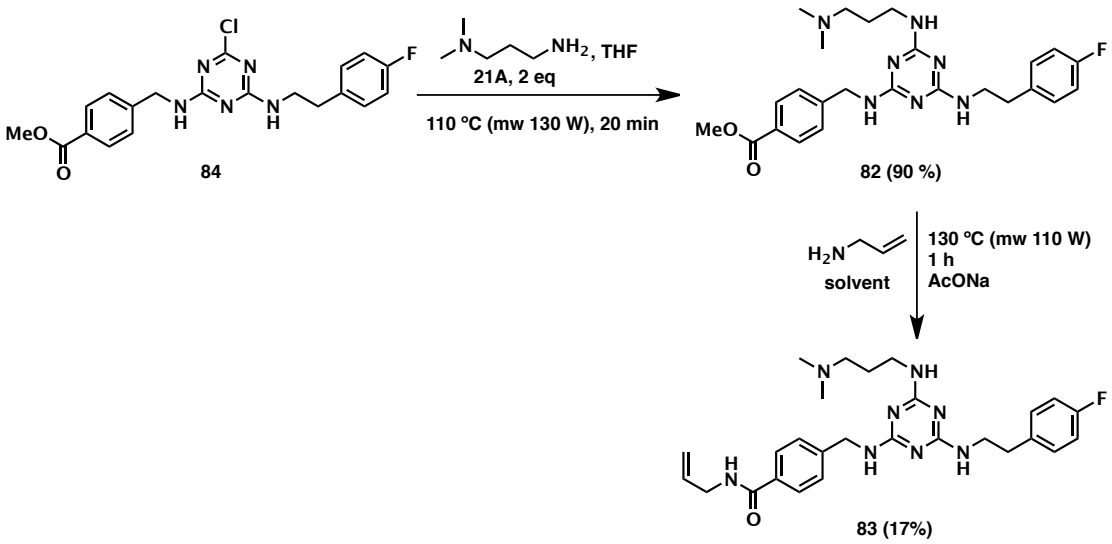


Figure 61. Synthesis of triazine derivatives **82** and **83**.

2.7 Optimization of the synthetic route for the preparation of gram scale amounts of 46.

The good results of TRPV1 antagonist activity obtained for triazine **46** during the course of the research project (Chapter 4), required that further determination of selectivity/toxicity profiles and *in vivo* evaluation were carried out. For that purpose, to improve the synthesis of **46** in order to obtain larger amounts of this triazine became an essential goal. The previously described method allowed the preparation of **46** in relatively moderate amounts (i.e. ~200 mg) per batch, but the amount required was on the order of at least 2 g with >95 % purity.

The sealed tubes used for the routine general synthesis of triazines were too small for the desired quantities, therefore the use of an open reactor coupled to a

refrigeration system was required for the synthesis of larger amounts of product and reaction intermediates.

Under these conditions, the first difference observed was that heat dissipation was worse in the open reaction vessel. Dropwise addition of reagents was not enough to maintain the low temperatures required to avoid undesired reactions and, therefore, cooling was required during the mix of reagents. In this context, the initial exothermic reaction between cyanuric chloride (**16**) and amine **20c** generated solid clusters in the reaction mixture. If the mixing was performed too slowly, in addition to the undesired temperature increase, formation of larger solid clusters were observed which could occlude reagents and byproducts. For these reasons, the best conditions found implied addition of amine **20c** to the solution of **16** outside of the microwave oven, in a cooling bath at 0 ºC. After the initial exothermic phase, the reaction vessel was placed in the microwave oven and two irradiation cycles were needed to achieve complete depletion of the monosubstituted **85** and conversion to **23** (Table 5).

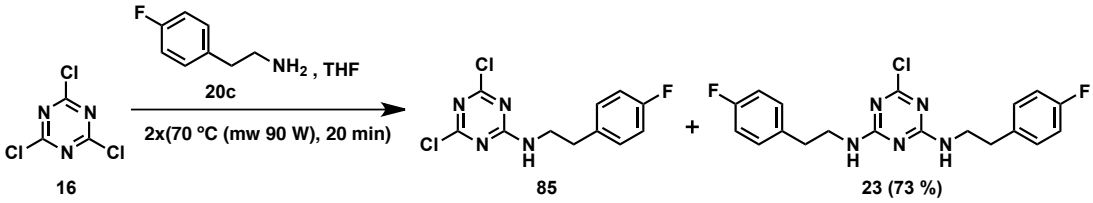


Figure 62. Synthetic scheme to obtain **23** in gram scale.

Table 5. Best reaction conditions for the preparation of **23** in gram scale.

Cycle	mmol 16	mmol 20c	Temp (ºC)	t (min)	P (W)	% area 85	% area 23
1	6	24	70	20	90	20	86
2	---	---	70	20	90	< 1	> 95

Purification of **23** was achieved using the protocol developed for disubstituted triazines and no further chromatographic purification was required, yielding **23** with a yield of 95 %.

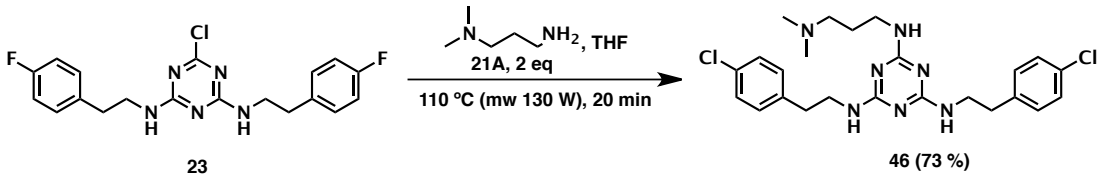


Figure 63. Synthesis of triazine **46** from **23**.

When a large amount of **23** (i.e. ~1 g) was allowed to react with an excess of **21A** using the general conditions for trisubstituted triazines, formation of undesired products, which were not identified, was observed and the best conversion towards the desired product was only ~60 %. Assays to determine the maximum amounts of reagents that could be used without modifying the protocol and minimising the side reactions were carried out. Thus, under the best conditions, up to ~400 mg of **23**. could be prepared per reaction batch. The standard work-up and chromatographic purification of the product was performed over the crude reaction mixtures of five batches, yielding 2,28 g of the desired triazine **23** with a purity above 99 % (HPLC), enough to perform the planned biological studies.