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ORIGINAL PAPER

Library synthesis of cardiomyogenesis inducing compounds using an efficient two-step-one-flow process

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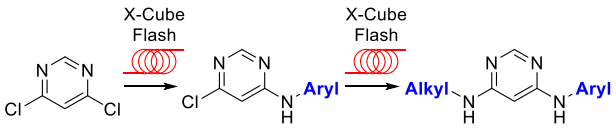
Abstract Within this work we telescoped a batch laboratory scale synthesis towards a two-step-one-flow process to synthesize cardiomyogenesis inducing compounds (4,6-diaminopyrimidines) in continuous manner. Special attention was put on a quick and robust screening protocol and subsequent UHPLC analysis. Finally, the robustness of the method was proven by the multi-gram synthesis of VUT-MK142 using a laboratory scale continuous flow reactor, enabling to deliver enough material for extensive biological testing.

Graphical abstract



Keywords Continuous flow chemistry · Medicinal chemistry · Library synthesis · Diaminopyrimidines

Introduction

In nature, cell differentiation is controlled by molecular processes which involve cell signaling [1]. Generally, cells differentiate from totipotent stem cells into more specialized multipotent cells until the final faith of the cell is reached with completely differentiated cells. This differentiation is growth-factor induced and relies on molecular level on signal transmission based on receptor binding. Consequently, changes in protein conformation and phosphorylation take place, which is equivalent to cell activation. This differential gene activity is responsible for the switch in the type of a cell [2].

There exist a number of possibilities to influence cell differentiation artificially. Complementary DNA fragments can be transferred into cells by retroviral vectors resulting in expression of the corresponding proteins. This technique was exploited to transfer specific transcription factors which consequently led to cell differentiation [3] and reprogramming [4], respectively. However, the risk of permanent changes in the genome and the usage of oncogenic transcription factors make this approach a controversial one. Another possibility to change the fate of a cell is nuclear transfer which is termed nuclear reprogramming [5, 6]. A very active field of research is the reprogramming of non-pluripotent somatic cells into induced pluripotent stem cells which was ultimately awarded with the Nobel Prize in Physiology or Medicine in 2012. This can be achieved by the artificial expression of several transcription factors wherefore a couple of methods exist [7–12]. A very promising approach towards a therapeutic application is an induced cell differentiation [13] and reprogramming [14] by synthetic small molecules (SySMs). Such cell fate controlling molecules attracted strong interest in recent years due to the potential and

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beneficial application for certain regenerative tissue therapies [13]. Among other differentiation processes, the transformation towards cardiomyocytes is particularly interesting as the heart has naturally poor regenerating features [15]. There are a couple of cardiomyogenesis inducing compounds known to the literature. Early examples are given by Wu et al. They found four 2,4-diaminosubstituted pyrimidines (cardiogenols A–D, Fig. 1) to be potentially able to induce cardiomyogenesis in murine embryonic stem cells. Thus, stem cells can be influenced to transform into cardiac muscle cells [16]. Other examples of cardiogenic SySMs include a 2,6-disubstituted 4-anilinoquinazoline derivative [17], Shz-1 [18], XAV939 [19], and CHIR99021 [20] (Fig. 1).

The diamino substituted pyrimidines synthesized by Wu et al. served as an inspiration for the preparation of similar compounds in our laboratory. To synthesize cardiogenol derivatives, a sequential nucleophilic substitution route was reported [21]. Furthermore, also 4,6-diaminopyrimidines as structural isomers to cardiogenols are known to derive from an accelerated microwave-assisted protocol [22] and flow-chemical synthesis of 2-aminopyrimidines was previously published [23]. Based on the structure of cardiogenol C, the previously reported compound VUT-MK142 (Fig. 2) was also found to be an efficient cardiomyogenesis inducing agent [24]. The compound was tested successfully on a mouse skeletal myoblast cell line (C2C12) and a mouse embryonic carcinoma cell line (P19) by three different assays giving remarkable transformation results on C2C12 cells: they could be transformed into functional cardiomyocytes, which was the first report of induced cardiomyogenic effects on lineage committed cells [25, 26]. The formation of cardiomyocytes from transplanted cells in the heart of a patient may improve the heart function significantly, e.g., after cardiac infarction. Therefore, such growth factors are of strong interest as heart diseases are the leading causes of death in the United States in 2011 [27].

To allow for extensive biological testing of 4,6-disubstituted pyrimidines and to evaluate the biological effects of VUT-MK142 derivatives, the known batch protocol was transferred into a continuous flow procedure which bears the potential for automated synthesis. Following this approach, a small library of differently substituted pyrimidines was synthesized, and a continuous multi-gram synthesis of VUT-MK142 was developed.

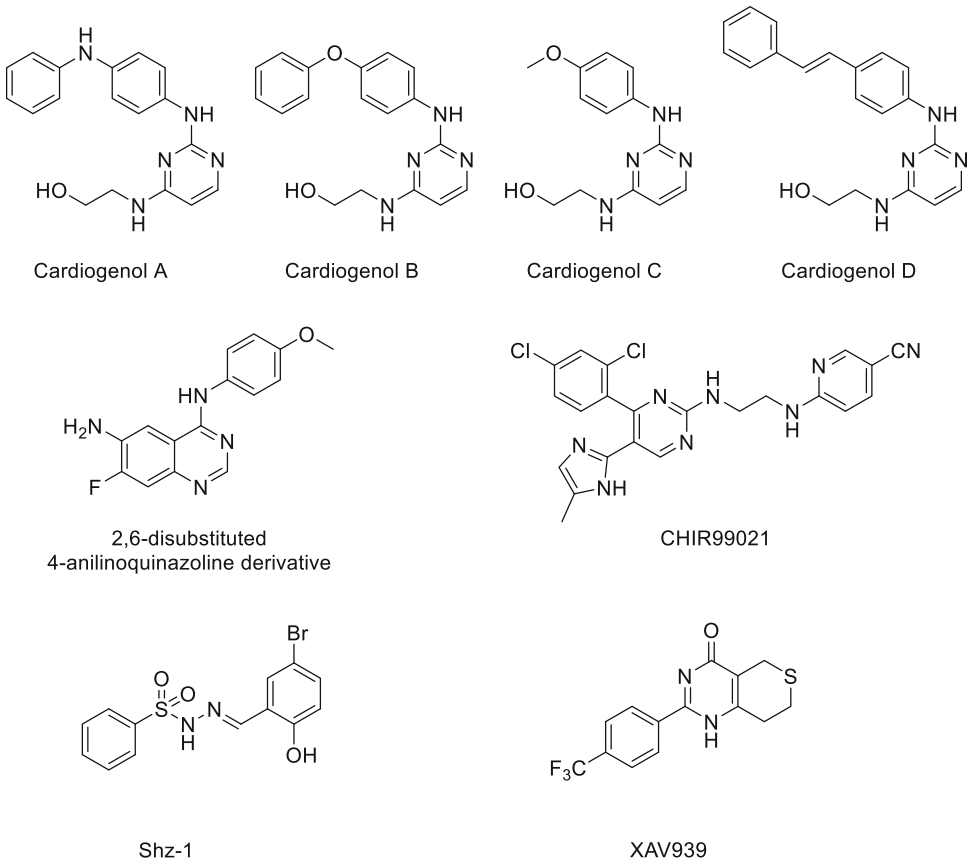


Fig. 1 Cardiomyogenesis inducing compounds

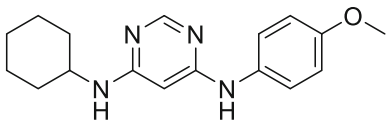


Fig. 2 VUT-MK142 as an example for 4,6-disubstituted pyrimidines inducing cardiomyogenesis

Results and discussion

The first milestone in the library synthesis of 4,6-disubstituted pyrimidines was the development of a model reaction sequence leading to VUT-MK142 as target product. Retrosynthetic analysis shows that VUT-MK142 could be synthesized via two sequential nucleophilic substitutions starting from 4,6-dichloropyrimidine (Scheme 1).

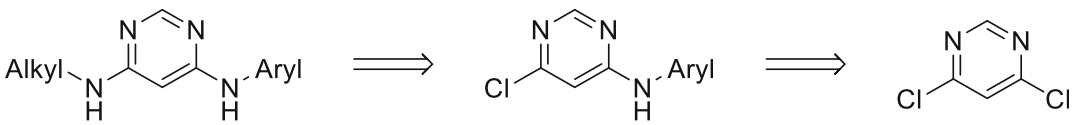
Due to the symmetry of 4,6-dichloropyrimidine, the less-reactive aromatic amine could be reacted first, followed by the second substitution reaction with an excess of cyclohexylamine as more reactive reagent. Selectivity for a mono-substitution should be achievable due to the de-activating nature of the newly introduced arylamine substituent. To allow for a quick, automation-assisted optimization of the two sequential substitution reactions, reference material was synthesized using a batch protocol (see "Experimental" section). Furthermore, an ultra-quick UHPLC method of 2 min was established to enable a rapid analysis of the screened reaction parameters (details see supplementary information).

In the first step, 4,6-dichloropyrimidine was reacted in flow with *p*-anisidine at 10 mM concentration in NMP under base catalysis at temperatures ranging from 150 to 200 °C in 10 °C increments (Scheme 2). The ThalesNano X-Cube Flash was chosen as reactor system, as it is available in a Hastelloy version which provides optimal chemical resistance, interchangeable reactor coils to influence residence times and, additionally, it allows very high reaction temperatures up to 350 °C—temperatures assumed to be beneficial in the second, less favored substitution step. Conversion was monitored to be incomplete in all cases with a maximum UHPLC yield of 44 %. Therefore, the concentration of the starting materials was increased to 50 and 100 mM boosting the conversion to completeness accompanied with an excellent maximum UHPLC yield of 97 % at 160 °C reaction temperature and 8 min of residence time in the reaction coil (Fig. 3, details see "Experimental" section).

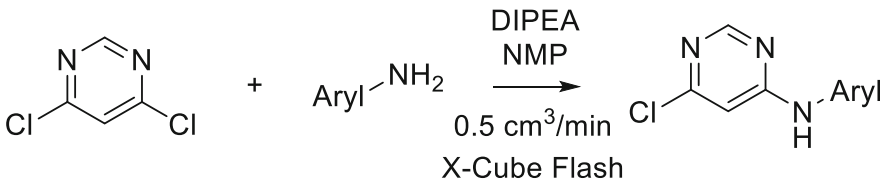
Interestingly, the highest temperature did not turn out to work best, but depending on the substrate concentration, 160 °C showed optimal reaction performance. Increasing the substrate concentration from 10 to 100 mM concentration increased the product yield from 44 % (160 °C, 10 mM) to 97 % (160 °C, 100 mM). An increase in reaction temperature again led to a drop in yields.

Subsequently, also the second substitution reaction was carried out in flow starting from purified intermediate of the first step (10 mM) and cyclohexylamine (30 mM) under base catalysis (Scheme 3). To compensate the reduced reactivity of the mono-substituted chloropyrimidine, the reaction temperature range of choice was increased to an interval of 200–300 °C with increments of 20 °C. Already in the first runs, the highest temperature of 300 °C was skipped due to repeated reactor cloggage. Furthermore, a limited stability of the desired target molecule at higher temperatures, as well as the occurrence of dechlorinated intermediate as by-product (identified via chromatography with a known reference compound as

Scheme 1



Scheme 2



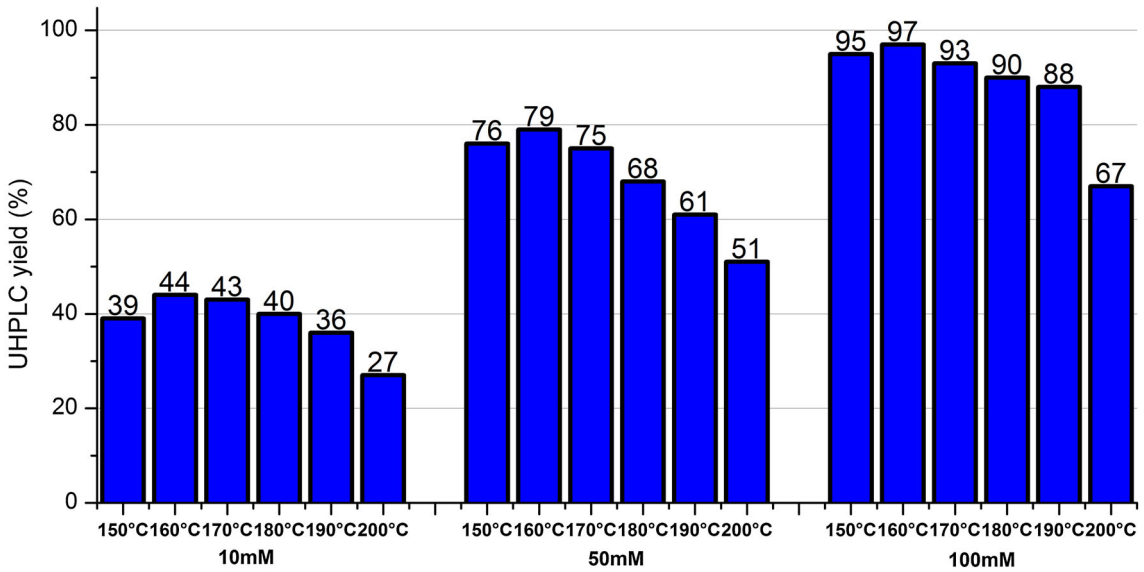
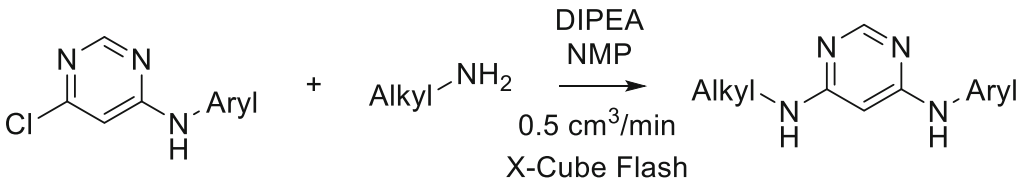


Fig. 3 UHPLC yields of the first reaction step at different concentrations and temperatures

Scheme 3



standard) was monitored, explaining the poor maximum yield of 25 % at 240 °C reaction temperature.

Following the strategy of the first substitution reaction, the reaction concentration was increased to 50/150 mM (chloropyrimidine/amine) and 100/300 mM giving significantly improved UHPLC yield of 79 % at 50 mM concentration (referenced to intermediate conversion) and 220 °C reaction temperature (Fig. 4). To boost the yield even more, we also increased the molar ratio of cyclohexylamine to intermediate from 3:1 up to a ratio of 10:1. This approach enabled us to get 94 % UHPLC yield at the lowest screened reaction temperature of 200 °C and 8 min residence time in the reaction coil (Figs. 5, 6), which was significantly higher than the yield of the corresponding batch reaction (38 %). Unfortunately, the combination of high substrate concentrations and high reaction temperatures led to an increased number of system cloggages, rendering a number of experiments impossible to perform (for details see "Experimental" section).

To summarize the optimization series, optimal conditions were identified to be 160 °C reaction temperature for the first reaction step and 200 °C temperature for the second nucleophilic substitution reaction. The reaction time of 8 min reaction time (which equaled a flow rate of 0.5 cm^3/min using a 4 cm^3 coil) was kept the same for all optimization reactions.

To give access to a small library of mono-substituted and 4,6-disubstituted pyrimidines, *p*-anisidine, *m*-anisidine, *m*-chloroaniline, and *o*-toluidine were reacted with 4,6-dichloropyrimidine according to the optimized reaction conditions in good to very good isolated yields (Table 1). Despite all optimization efforts, the reaction time had to be doubled for the latter three amines doubling the size of the reaction coil to 8 cm^3 to give full conversion of starting materials (see "Experimental" section).

In the synthesis of the desired target molecules, it was exemplarily shown that the use of purified intermediate gave VUT-MK142 in very good isolated yield of 84 %, which equaled to a total yield over two steps of 68 % (see "Experimental" section). However, merging the two reaction steps into a single reaction sequence without intermediate purification could further streamline the synthesis of the target compounds by reducing the purification steps to a single one. After the first substitution reaction, a

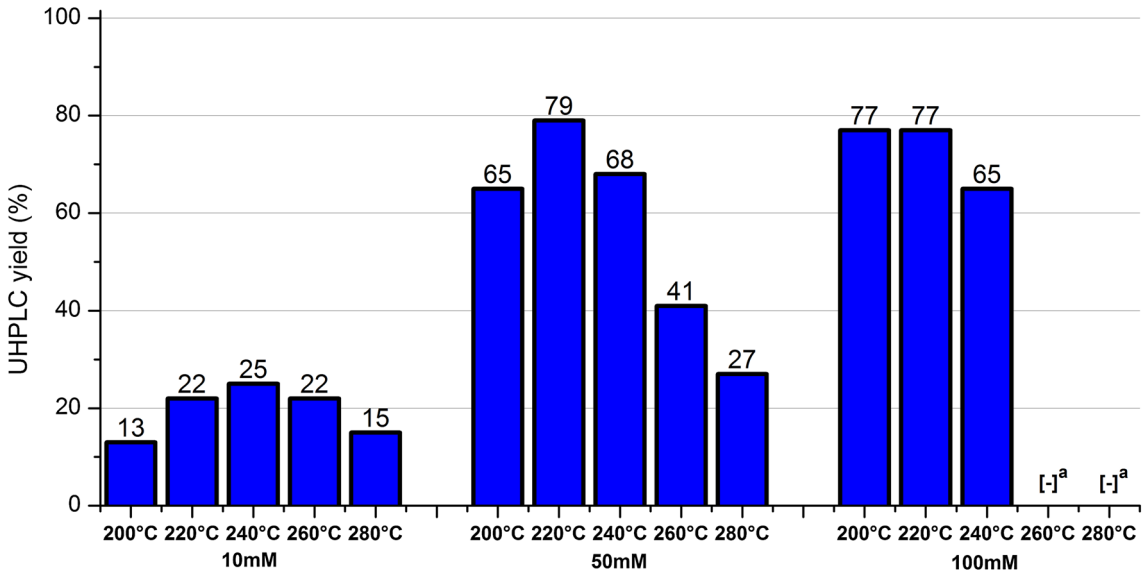


Fig. 4 UHPLC yields of the second reaction step at different concentrations and temperatures; 3 equiv. cyclohexylamine. *Superscript a* reaction conditions led to system cloggage

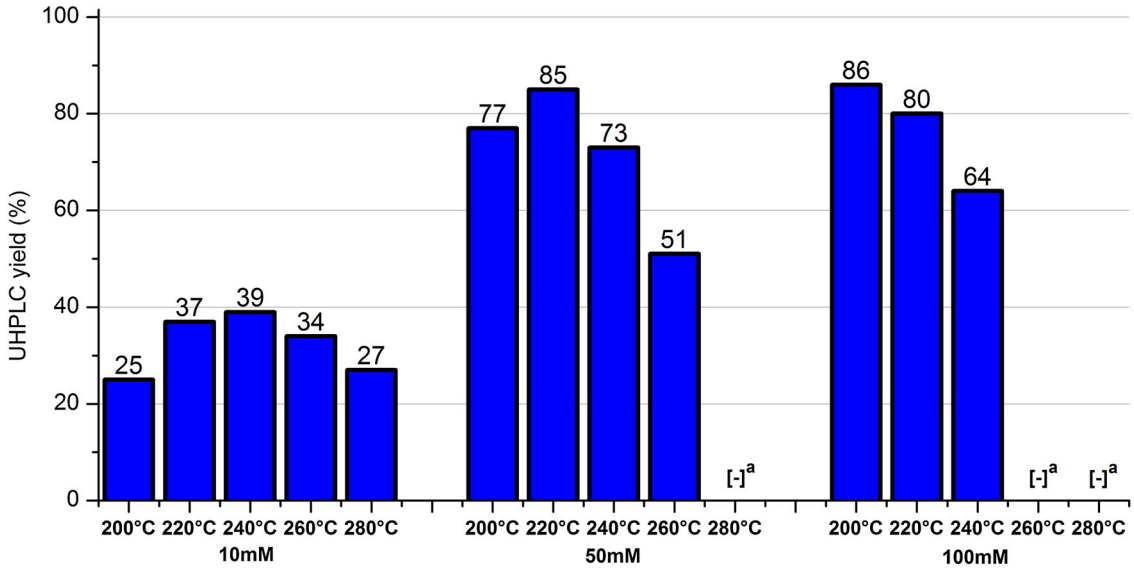


Fig. 5 UHPLC yields of the second reaction step at different concentrations and temperatures; 5 equiv. cyclohexylamine. *Superscript a* reaction conditions led to system cloggage

defined aliquot was mixed with the appropriate amount of cyclohexylamine and subjected to the reactor system for a second time. Due to this fact, synthesis of all 4,6-disubstituted pyrimidines was carried out following the single reaction sequence giving products in acceptable to good yields (Table 2, for details see "Experimental" section).

Finally, it was also demonstrated that the prevalent method was also suitable to synthesize VUT-MK142 on large scale. To double the amount of product keeping the specific reaction parameters constant for this specific reaction sequence, the coil size was doubled from 4 to 8 cm^3, as well as the flow rate from 0.5 to 1.0 cm^3/min. Allowing the system to run for about 4 h, more than 5 g of pure product could be produced still using a laboratory scale flow reactor, which equals a productivity of 29.1 g/day.

Conclusion

In this work, we successfully transferred the previously published synthesis of the cardiomyogenesis inducing compound VUT-MK142 into a continuous flow process. The reaction was optimized to enable a high yield synthesis

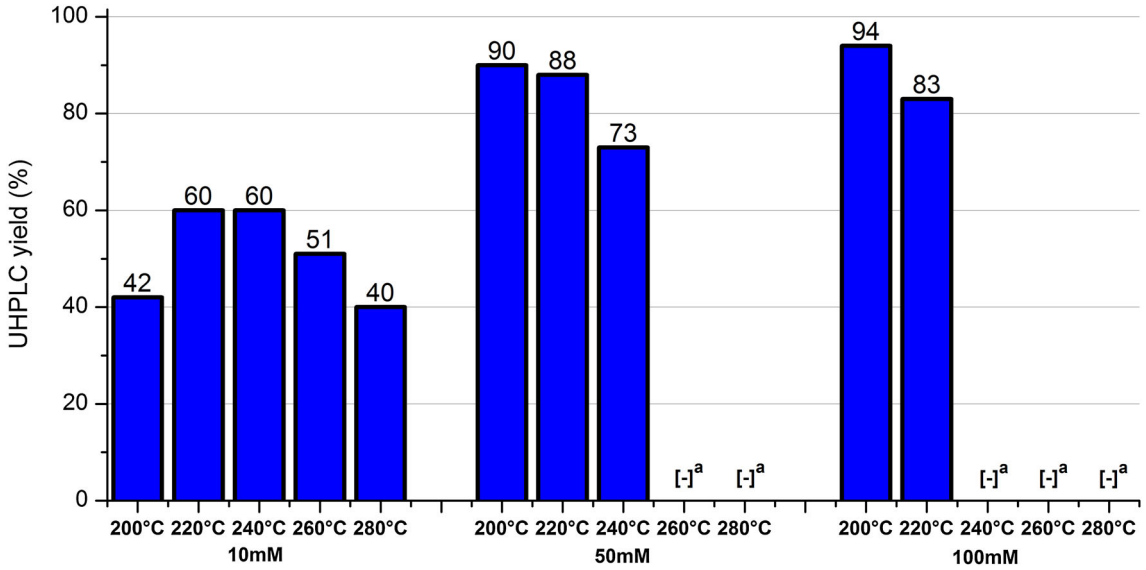
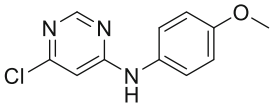
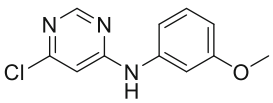
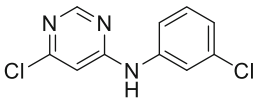
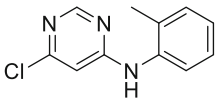


Fig. 6 UHPLC yields of the second reaction step at different concentrations and temperatures; 10 equiv. cyclohexylamine. *Superscript a* reaction conditions led to system cloggage

Table 1 Library synthesis of mono-substituted chloropyrimidines in flow

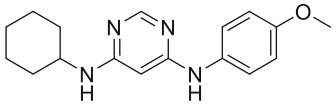
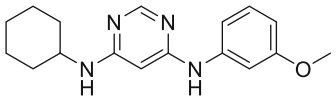
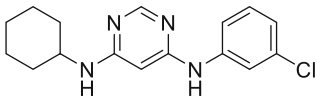
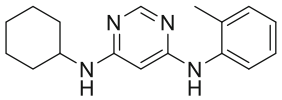
Product	Structure	Isolated yield /%
1		81
2		60
3		51
4		53

with an effective residence time in the range of minutes. The selection of a sequential amination approach allowed for an increased yield compared to the batch protocol and lowered the work-up effort. Consequently, the method was telescoped to give a productivity of 29.1 g/day. To broaden the scope of the reaction, a small library of intermediates and diaminopyrimidine products was successfully synthesized without major changes to the standard protocol.

Experimental

Solvents: NMP, DMA, DMF, and DMSO were purchased from Merck, Sigma-Aldrich, or Loba. Starting materials: 4,6-dichloropyrimidine, *p*-anisidine, *m*-anisidine, *m*-chloroaniline, *o*-toluidine, *N*,*N*-diisopropylethylamine, and cyclohexylamine were bought from Sigma-Aldrich, Fluka, Merck, or Baker. During work-up, diisopropylether, chloroform, sodium chloride, ammonium chloride, sodium carbonate, and benzyl benzoate were supplied by Acros, Neuber, and Merck. For chromatography, redistilled petroleum ether and ethyl acetate were used, running on Merck silica gel. ^{1}H and ^{13}C NMR spectra were recorded using TMS as an internal standard on a Bruker Avance 200 MHz spectrometer. Chemical shifts were reported in ppm. Reactions in flow were performed on ThalesNano® X-Cube Flash high temperature/high pressure reactor. As a reaction coil, 4 and 8 cm^3 Hastelloy® coils were installed. To precisely collect the product plug in the screening process, the dead volume of the system and the heat exchangers was determined for each coil using an intensively colored solution. Reaction analysis was carried out using a Shimadzu Nexera® UHPLC system using a Phenomenex Kinetex® PFP column for separation running on an acetonitrile–water gradient (details see supplementary information). To guarantee for robust measurements, an internal standard protocol was established. Product purification was performed on a Büchi Sepacore® MPLC system. Melting points were measured on a calibrated Stanford Research Systems Optimelt MPA100 automated melting point system.

Table 2 Library and large scale synthesis of 4,6-disubstituted pyrimidines in flow

Product	Structure	Isolated yield /% (single step)
5 (VUT-MK-142)		70 (68 with purified intermediate)
6		48
7		42
8		51

General protocol A

The following general protocol A was used for the optimization of the flow synthesis and preparation of 4-substituted pyrimidines in flow: reaction lines 1 and 2 were purged with pure NMP to get rid of air bubbles. Then, reaction line 1 was changed to starting material solution. The flow rate was set to 0.5 cm^3/min and the backpressure was set to 75 bar (to have sufficient tolerance in the case of pressure fluctuations). Reaction temperature was set to the desired value and all the conditions were allowed to stabilize for 10 min running on solvent only. Then, the pumps were switched to introduce the starting material solution into the system. After completion, the pumps were switched back to NMP, dead volume was allowed to pass and subsequent product collection was triggered. Using this protocol, reaction temperature as well as concentration and molar ratio of starting materials were optimized. In the case of incomplete conversions, size of the reaction coil was changed from 4 to 8 cm^3 (instead of varying the flow rates) so as not to affect the space–time-yield of the process. To increase productivity and minimize product decomposition, in some cases the flow rate was increased from 0.5 to 1.0 cm^3/min.

General protocol B

In the case of 4,6-diaminopyrimidine derivative one-flow synthesis, general protocol B was developed: cyclohexylamine and base were added to the reaction solution after the first step without further purification. To take account for dilution effects caused by addition of reagents and diffusion of the reaction plug during the first reaction, starting material concentration of the first reaction was increased to about 120 mM 4,6-dichloropyrimidine and 132 mM *N*,*N*-diisopropylethylamine as a compensation. System parameters were equal to general protocol A.

Reference material synthesis

*6-Chloro-N-(4-methoxyphenyl)pyrimidine-4-amine (**1**)*
In batch: 317 mg 4,6-dichloropyrimidine (2.13 mmol, 1.0 equiv.), 262 mg *p*-anisidine (2.13 mmol, 1.0 equiv.), and 303 mg *N*,*N*-diisopropylethylamine (2.34 mmol, 1.1 equiv.) were introduced into a high pressure glass vial, equipped with a magnetic stirring bar and dissolved in 15 cm^3 NMP. The reaction solution was heated conventionally to 120 °C for 10 h. After cooling, 25 cm^3 EtOAc was added and the solution was extracted three times with saturated solution of ammonium chloride (3 × 25 cm^3). The combined aqueous phases were extracted once again with 50 cm^3 EtOAc and the combined organic phases were washed thoroughly with brine (5 × 25 cm^3). After drying the organic phase with anhydrous sodium sulfate, filtration, and evaporation of the solvent, crude product was obtained as a brown solid. Further purification using chromatography gave pure product **1** as a white, fluffy solid in 75 % yield (380 mg, 1.61 mmol). Spectral data were in accordance with literature [25].

*N^4-Cyclohexyl-N^6-(4-methoxyphenyl)pyrimidine-4,6-diamine (**5**)*
In batch: 49 mg 4,6-dichloropyrimidine (0.33 mmol, 1.0 equiv.), 41 mg *p*-anisidine (0.33 mmol, 1.0 equiv.), and 47 mg *N*,*N*-diisopropylethylamine (0.37 mmol, 1.0 equiv.) were reacted as described above. The reaction solution was

used for the second reaction step without further purification. Cyclohexylamine (99 mg, 1.00 mmol, 3.0 equiv.) and 129 mg *N*,*N*-diisopropylethylamine (1.00 mmol, 3.0 equiv.) were added and the reaction solution was heated to 200 °C for 17 h. Work-up and purification was carried out as for the intermediate compound giving pure product **5** as a white, fluffy solid in 38 % yield (38 mg, 0.13 mmol). Spectral data were in accordance with literature [25].

First step optimization in flow

As a starting point for the synthesis, a solution of 74 mg 4,6-dichloropyrimidine (0.50 mmol, 1.0 equiv.), 62 mg *p*-anisidine (0.50 mmol, 1.0 equiv.), and 71 mg *N*,*N*-diisopropylethylamine (0.55 mmol, 1.1 equiv) in 50 cm^3 NMP was prepared. For each optimization step, 4 cm^3 of starting material solution was introduced into the system (4 cm^3 coil) and reacted according to general procedure A at room temperature and 150–200 °C in 10 °C increments at a flow rate of 0.5 cm^3/min. To improve the conversion rates, starting material concentration was increased to 50 and 100 mM (based on 4,6-dichloropyrimidine), running at the same molar ratio and reaction conditions. The progress of the reaction optimization was monitored using UHPLC measurements.

Second step optimization in flow

To tune the parameters of the second reaction step, a starting material solution of 118 mg **1** (0.50 mmol, 1.0 equiv.), 149 mg cyclohexylamine (1.50 mmol, 3.0 equiv.), and 194 mg *N*,*N*-diisopropylethylamine (1.50 mmol, 3.0 equiv.) were dissolved in 50 cm^3 NMP. For each optimization step, 4 cm^3 of starting material solution was introduced into the system (4 cm^3 coil) and reacted according to general procedure B at room temperature and 200–280 °C in 20 °C increments at a flow rate of 0.5 cm^3/min. In the optimization series, cyclohexylamine molar ratio was increased to 5.0 and 10.0 equiv. and reaction solution concentration was increased to 50 and 100 mM (based on **1**). Due to enhanced fluctuation in the system pressure and increased number of system blockages, the highest screening temperature was lowered stepwise from 280 to 220 °C. The progress of the reaction optimization was monitored using UHPLC measurements.

Synthesis of 4-substituted pyrimidines in flow

*6-Chloro-N-(4-methoxyphenyl)pyrimidine-4-amine (**1**)*
4,6-Dichloropyrimidine (190 mg, 1.28 mmol, 1.0 equiv.), 157 mg *p*-anisidine (1.27 mmol, 1.0 equiv.), and 180 mg *N*,*N*-diisopropylethylamine (1.39 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.7 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using a 4 cm^3 coil at a flow rate of 0.5 cm^3/min. Crude product was purified as described for the batch synthesis giving pyrimidine **1** as a white, fluffy solid in 81 % yield (243 mg, 1.03 mmol).

*6-Chloro-N-(3-methoxyphenyl)pyrimidine-4-amine (**2**)*
4,6-Dichloropyrimidine (190 mg, 1.28 mmol, 1.0 equiv.), 157 mg *m*-anisidine (1.27 mmol, 1.0 equiv.), and 180 mg *N*,*N*-diisopropylethylamine (1.39 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.7 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 0.5 cm^3/min. To improve the work-up process, NMP was removed under high vacuum, the residue was dissolved in 25 cm^3 chloroform and extracted with a saturated solution of sodium carbonate (3 × 25 cm^3). Each aqueous phase was re-extracted with 10 cm^3 chloroform. The combined organic phases were washed with brine (3 × 25 cm^3). After drying the organic phase with anhydrous sodium sulfate, filtration, and evaporation of the solvent, crude product was obtained as a brown oil. Further purification using chromatography gave pure product **2** as a light brown solid in 60 % yield (180 mg, 0.77 mmol). Spectral data were in accordance with literature [25].

*6-Chloro-N-(3-chlorophenyl)pyrimidine-4-amine (**3**)*
4,6-Dichloropyrimidine (186 mg, 1.25 mmol, 1.0 equiv.), 160 mg *m*-chloroaniline (1.25 mmol, 1.0 equiv.), and 178 mg *N*,*N*-diisopropylethylamine (1.38 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.5 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate 0.5 cm^3/min. After product purification described in the synthesis of **2**, pure product **3** was isolated as a white solid in 51 % yield (153 mg, 0.64 mmol). Spectral data were in accordance with literature [25].

6-Chloro-N-(2-methylphenyl)pyrimidine-4-amine
(**4**, $C_{11}H_{10}ClN_3$)
4,6-Dichloropyrimidine (203 mg, 1.36 mmol, 1.0 equiv.), 145 mg *o*-toluidine (1.35 mmol, 1.0 equiv.), and 194 mg *N*,*N*-diisopropylethylamine (1.50 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 13.6 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 0.5 cm^3/min. After product purification described in the synthesis of **2**, pure product **4** was isolated as yellow crystals in 53 % yield (158 mg, 0.72 mmol). M.p.: 121–123 °C; ^{1}H NMR (DMSO-d_6, 200 MHz): $\delta = 2.18$ (s, 3H), 6.53 (s, 1H),

7.02–7.47 (m, 4H), 8.33 (s, 1H), 9.35 (br, 1H) ppm; ^{13}C NMR (DMSO-d_6, 50 MHz): $\delta = 17.8$ (q), 103.0 (d), 125.9 (d), 126.1 (d), 126.5 (d), 130.8 (d), 133.3 (s), 136.1 (s), 158.3 (s), 158.6 (d), 162.5 (s) ppm; TLC (PE:EtOAc = 1:1): $R_f = 0.59$.

Synthesis of 4,6-disubstituted pyrimidines in flow using purified starting materials

*N^4-Cyclohexyl-N^6-(4-methoxyphenyl)pyrimidine-4,6-diamine (**5**)*

1 (237 mg, 1.01 mmol, 1.0 equiv.), 998 mg cyclohexylamine (10.06 mmol, 10.0 equiv.), and 390 mg *N*,*N*-diisopropylethylamine (3.02 mmol, 3.0 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 10.1 cm^3 with NMP. The solution was reacted according to general procedure A at 200 °C using a 4 cm^3 coil at a flow rate of 0.5 cm^3/min. EtOAc (25 cm^3) was added to the reaction solution. By adding 25 cm^3 of a saturated solution of sodium carbonate, a precipitate appeared in the organic phase, which could be identified as product. EtOAc was added until the organic phase was homogeneous again and the organic layer was extracted with a saturated solution of sodium carbonate (2 × 25 cm^3). The combined aqueous phases were further extracted with EtOAc (3 × 25 cm^3) and the organic layers were washed with brine (3 × 25 cm^3). After drying the organic phase with anhydrous sodium sulfate, filtration, and evaporation of the solvent, crude product was obtained as a brown oil. Further purification using chromatography gave pure product **5** as a white, fluffy solid in 84 % yield (251 mg, 0.84 mmol).

Synthesis of 4,6-disubstituted pyrimidines in flow using a one-flow process

*N^4-Cyclohexyl-N^6-(4-methoxyphenyl)pyrimidine-4,6-diamine (**5**)*

4,6-Dichloropyrimidine (214 mg, 1.44 mmol, 1.0 equiv.), 177 mg *p*-anisidine (1.44 mmol, 1.0 equiv.), and 204 mg *N*,*N*-diisopropylethylamine (1.58 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.0 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using a 4 cm^3 coil at a flow rate of 0.5 cm^3/min. Then, an aliquot of 4.5 cm^3 reacted solution (max. 0.54 mmol, 1.0 equiv.) was taken for the second reaction step, and 534 mg cyclohexylamine (5.39 mmol, 10.0 equiv.) and 209 mg *N*,*N*-diisopropylethylamine (1.62 mmol, 3.0 equiv.) were added. The solution was reacted according to general procedure B at 200 °C using a 4 cm^3 coil at a flow rate of 0.5 cm^3/min. After product purification described in the synthesis of **2**, pure product **5** was isolated as a white solid in 70 % yield (113 mg, 0.38 mmol).

*N^4-Cyclohexyl-N^6-(3-methoxyphenyl)pyrimidine-4,6-diamine (**6**)*

4,6-Dichloropyrimidine (214 mg, 1.44 mmol, 1.0 equiv.), 177 mg *m*-anisidine (1.44 mmol, 1.0 equiv.), and 204 mg *N*,*N*-diisopropylethylamine (1.58 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.0 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 0.5 cm^3/min. Then, an aliquot of 8.4 cm^3 reacted solution (max. 1.01 mmol, 1.0 equiv.) was taken for the second reaction step and 997 mg cyclohexylamine (10.05 mmol, 10.0 equiv.) and 390 mg *N*,*N*-diisopropylethylamine (3.02 mmol, 3.0 equiv.) were added. The solution was reacted according to general procedure B at 200 °C using an 8 cm^3 coil at a flow rate of 1.0 cm^3/min. After evaporating NMP under high vacuum, the residue was directly purified by MPLC giving pure product **6** as a light brown solid in 48 % yield (143 mg, 0.48 mmol). Spectral data were in accordance with literature [25].

*N^4-Cyclohexyl-N^6-(3-chlorophenyl)pyrimidine-4,6-diamine (**7**)*

4,6-Dichloropyrimidine (214 mg, 1.44 mmol, 1.0 equiv.), 184 mg *m*-chloroaniline (1.44 mmol, 1.0 equiv.), and 204 mg *N*,*N*-diisopropylethylamine (1.58 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.0 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 0.5 cm^3/min. Then, an aliquot of 8.3 cm^3 reacted solution (max. 0.99 mmol, 1.0 equiv.) was taken for the second reaction step and 984 mg cyclohexylamine (9.92 mmol, 10.0 equiv.) and 384 mg *N*,*N*-diisopropylethylamine (2.97 mmol, 3.0 equiv.) were added. The solution was reacted according to general procedure B at 200 °C using an 8 cm^3 coil at a flow rate of 1.0 cm^3/min. After product purification described in the synthesis of **2**, pure product **7** was isolated as a light brown solid in 42 % yield (126 mg, 0.42 mmol). Spectral data were in accordance with literature [25].

N^4-Cyclohexyl-N^6-(2-methylphenyl)pyrimidine-4,6-diamine (**8**, $C_{17}H_{22}N_4$)

4,6-Dichloropyrimidine (214 mg, 1.44 mmol, 1.0 equiv.), 154 mg *o*-toluidine (1.44 mmol, 1.0 equiv.), and 204 mg *N*,*N*-diisopropylethylamine (1.58 mmol, 1.1 equiv.) were introduced into a Falcon® tube, dissolved in NMP, and filled up to a volume of 12.0 cm^3 with NMP. The solution was reacted according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 0.5 cm^3/min.

Then, an aliquot of 8.9 cm^3 reacted solution (max. 1.07 mmol, 1.0 equiv.) was taken for the second reaction step and 1.06 g cyclohexylamine (10.7 mmol, 10.0 equiv.) and 413 mg *N*,*N*-diisopropylethylamine (3.20 mmol, 3.0 equiv.) were added. The solution was reacted according to general procedure B at 200 °C using an 8 cm^3 coil at a flow rate of 1.0 cm^3/min. After product purification described in the synthesis of **2**, pure product **8** was isolated as a white solid in 51 % yield (153 mg, 0.54 mmol). M.p.: 219.5–221.5 °C; ^{1}H NMR ($CDCl_3$:CD_3OD = 3:1, 200 MHz): δ = 0.92–1.91 (m, 10H), 2.16 (s, 3H), 3.06–3.29 (m, 1H), 3.82 (br, 2H), 5.23 (s, 1H), 6.98–7.23 (m, 4H), 7.92 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$:CD_3OD = 3:1, 50 MHz): δ = 17.6 (q), 24.5 (t), 25.4 (t), 32.6 (t), 49.6 (d), 80.9 (d), 125.4 (d), 126.1 (d), 126.7 (d), 131.1 (d), 133.5 (s), 136.4 (s), 157.5 (d), 161.3 (s), 161.8 (s) ppm; TLC (PE:EtOAc = 1:1): R_f = 0.34.

Large scale synthesis of N^4-cyclohexyl-N^6-(4-methoxyphenyl)pyrimidine-4,6-diamine (**5**)

4,6-Dichloropyrimidine (3.97 g, 26.7 mmol, 1.0 equiv.), 3.28 g *p*-anisidine (26.7 mmol, 1.0 equiv.), and 3.79 g *N*,*N*-diisopropylethylamine (29.3 mmol, 1.1 equiv.) were dissolved in NMP and filled up to a volume of 220 cm^3 with NMP. The solution was prepared according to general procedure A at 160 °C using an 8 cm^3 coil at a flow rate of 1.0 cm^3/min. To the reacted solution (max. 26.7 mmol, 1.0 equiv.), 26.4 g cyclohexylamine (267 mmol, 10.0 equiv.) and 10.3 g *N*,*N*-diisopropylethylamine (80.0 mmol, 3.0 equiv.) were added. The solution was reacted according to general procedure B at 200 °C using an 8 cm^3 coil at a flow rate of 1.0 cm^3/min. NMP was evaporated under high vacuum, residue was dissolved in 300 cm^3 chloroform and extracted with a saturated solution of sodium carbonate (3 × 300 cm^3). Each aqueous phase was re-extracted with chloroform (3 × 30 cm^3) and the combined organic layers were washed with brine (3 × 300 cm^3). After drying the organic phase with anhydrous sodium sulfate, filtration, and evaporation of the solvent, crude product was obtained as a brown oil. The residue was dissolved in 180 cm^3 diisopropylether under reflux, which led to the precipitation of a bright solid. The solid was isolated by filtration and dried in vacuum. The solvents were evaporated and a second product fraction was obtained by recrystallization with 100 cm^3 EtOAc. Pure product **5** was isolated as a white solid in 64 % yield (5.06 g, 17.0 mmol). Note: Short periods of destabilized reaction conditions led to lower conversion, formation of N^4-,N^6-dicyclohexylpyrimidine-4,6-diamine as a by-product and thus a lower product yield as in the small scale experiment.

References

1. Mohammad HP, Baylin SB (2010) Nat Biotechnol 28:1033
2. Lodish H, Berk A, Kaiser C, Krieger M, Scott M, Bretscher A, Ploegh H, Matsudaira P (2008) Molecular Cell Biology, 6th edn. Freeman, New York
3. Davis RL, Weintraub H, Lassar AB (1987) Cell 51:987
4. Kulessa H, Frampton J, Graf T (1995) Genes Dev 9:1250
5. Gurdon JB, Byrne JA, Simonsson S (2003) Proc Natl Acad Sci USA 100:11819
6. Gurdon JB, Melton DA (2008) Science 322:1811
7. Maherali N, Hochedlinger K (2008) Cell Stem Cel 3:595
8. Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K (2008) Proc Natl Acad Sci USA 105:2883
9. Park I-H, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ (2008) Nature 451:141
10. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Cell 131:861
11. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Science 318:1917
12. Takahashi K, Yamanaka S (2006) Cell 126:663
13. Firestone AJ, Chen JK (2010) ACS Chem Biol 5:15
14. Feng B, Ng J-H, Heng J-CD, Ng H-H (2009) Cell Stem Cell 4:301
15. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F, Nurzynska D, Torella D, Rotatori F, Rastaldo R, Musso E, Quaini F, Leri A, Kajstura J, Anversa P (2005) Circ Res 97:663
16. Wu X, Ding S, Ding Q, Gray NS, Schultz PG (2004) J Am Chem Soc 126:1590
17. Shen G, Hu Y, Wu J, Jin K, Zhu D, Zhang Y, Yu Y, Lou Y (2012) ChemMedChem 7:733
18. Sadek H, Hannack B, Choe E, Wang J, Latif S, Garry MG, Garry DJ, Longgood J, Frantz DE, Olson EN, Hsieh J, Schneider JW (2008) Proc Natl Acad Sci USA 105:6063
19. Wang H, Hao J, Hong CC (2011) ACS Chem Biol 6:192
20. Gonzalez R, Lee JW, Schultz PG (2011) Angew Chem Int Ed 50:11181
21. Wu X, Ding S, Schultz PG (2005) 2,4-Diaminopyrimidines as inducers of cardiomyogenesis, their preparation and use in the treatment of cardiomyopathy. PCT Int. Appl. WO 2005068437 A1 20050728, Jul 28, 2005; (2005) Chem Abstr 143:172888
22. Hartung CG, Backes AC, Felber B, Missio A, Philipp A (2006) Tetrahedron 62:10055
23. Ingham RJ, Riva E, Nikbin N, Baxendale IR, Ley SV (2012) Org Lett 14:3920
24. Koley M, Mike AK, Heher P, Koenig X, Schön M, Schnürch M, Hilber K, Weitzer G, Mihovilovic MD (2013) Med Chem Commun 4:1189
25. Mihovilovic MD, Schnürch M, Koley M, Hilber K, Koenig X (2011) Preparation of phenylaminopyrimidinylamine derivatives and analogs for use as cardiovascular agents. PCT Int. Appl. WO 2011079343 A2 20110707, Jul 7, 2011; (2011) Chem Abstr 155:152546
26. Mike AK, Koenig X, Koley M, Heher P, Wahl G, Rubi L, Mihovilovic MD, Schnürch M, Weitzer G, Hilber K (2014) Cell Physiol Biochem 33:205
27. Hoyert DL, Xu J (2012) Natl Vital Stat Rep 61:1