

Rapid preparation of the mitotic kinesin Eg5 inhibitor monastrol using controlled microwave-assisted synthesis

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We present here a protocol for the synthesis of the dihydropyrimidine (DHPM) derivative monastrol, which is known to be a specific mitotic kinesin Eg5 inhibitor. By applying controlled microwave heating under sealed-vessel conditions, the synthesis via the one-pot three-component Biginelli condensation can be performed in a shorter reaction time (30 min) compared with conventional heating methods that normally require several hours of reflux heating. For the purification of the crude target compound, two different methods are presented. The first protocol includes a simple precipitation/filtration step to provide monastrol in 76% isolated yield and high purity so that no recrystallization step is necessary. This can be ascribed to the microwave heating technology in which less side-product formation is typically one of the advantages. In an alternative purification step, column chromatography is performed, which provides the product in a slightly higher yield (86%). Monastrol synthesis can be conducted in ~2 h by employing the precipitation/filtration purification method.

INTRODUCTION

By screening a 16,320-member library of diverse small molecules in 1999, Mayer *et al.* have identified racemic monastrol (**1**, see Fig. 1), 4-(3-hydroxyphenyl)-3,4-dihydropyrimidine-2(1H)-thione, as a novel, cell-permeable compound for the development of potentially new anticancer drugs¹. In contrast to other anticancer drugs that perturb mitosis by binding to the protein tubulin-like natural taxanes, vinca alkaloids and epothilones^{1,2}, monastrol specifically affects the cell division by a new mechanism. The discovery of a new class of proteins, the mitotic kinesins, offers a novel approach to cancer treatment. These proteins are exclusively involved in the formation and function of the bipolar mitotic spindle. It was established that monastrol blocks mitosis by specific and reversible inhibition of the motor activity of the mitotic kinesin Eg5. This leads to the cells having monopolar spindles and cell cycle arrest during mitosis. Recently, the groups of Surrey and Giannis have reported on the synthesis of the monastrol analogs enastron (**2**) and dimethylenastron (**3**, see Fig. 1)³. Enastron proved to be ten times more potent than monastrol, and dimethylenastron showed an even greater—100 times higher—inhibition activity against Eg5. Since the original discovery of monastrol, several other mitotic kinesin Eg5 inhibitors have been reported⁴.

In recent years, microwave-assisted organic synthesis (MAOS) has attracted considerable attention, and it has become a popular and convenient tool for performing organic reactions at high speed^{5–9}. In particular, the use of dedicated microwave reactors (see Fig. 2) that enable the rapid and safe heating of reaction mixtures in sealed vessels under controlled conditions with online temperature and pressure monitoring has greatly increased the general acceptance of this method. Not only is direct microwave heating often able to reduce reaction times, but it is also known to reduce side reactions, increase yields and improve reproducibility, when compared with conventional thermal heating. Most of the recently described microwave procedures are performed under closed-vessel conditions, since most of the commercially available

microwave reactors are designed for this purpose. This has the advantage that reactions can be performed far above the boiling point of the solvent at elevated pressures, which typically leads to reduced reaction times (from hours to minutes and even seconds) and improved yields^{5–9}.

A direct and simple method for the synthesis of dihydropyrimidines (DHPMs) is the Biginelli multicomponent reaction, a one-pot cyclocondensation of a β -ketoester, aldehyde and (thio)urea under acidic conditions, which was first reported by Biginelli in 1893 (see Fig. 3)^{10,11}. The obtained multifunctionalized DHPM derivatives exhibit a broad range of interesting pharmacological properties¹² apart from their function as mitotic kinesin Eg5 inhibitors.

For the synthesis of the DHPM derivative monastrol (**1**), we decided on a microwave-assisted protocol under closed-vessel conditions employing the Biginelli cyclocondensation of ethyl acetoacetate (**4**), 3-hydroxybenzaldehyde (**5**) and thiourea (**6**), which is depicted in Figure 3. Since it is known that conventional heating methods for this reaction normally require several hours of reflux heating, we wanted to take advantage of direct and rapid microwave dielectric heating to enhance the reaction rate and to obtain a higher purity of the DHPM product (see Box 1 for the advantages of microwave heating). For this particular Biginelli

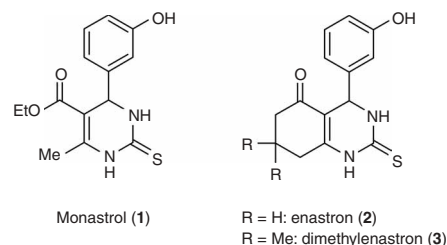


Figure 1 | Mitotic kinesin Eg5 inhibitors.

condensation, the best results were obtained using ytterbium(III) trifluoromethanesulfonate, Yb(OTf)₃, as Lewis acid catalyst and acetonitrile (MeCN) as solvent^{13,14}. We have used a similar protocol employing EtOH as solvent for the generation of a 48-member DHPM compound library in 2001, applying automated sequential microwave synthesis¹³. Since the original 1999 report¹, which did not disclose the synthesis of monastrol, several methods using different catalysts have been described for the preparation of monastrol via Biginelli three-component condensation under both conventional and microwave heating conditions. The published methods reported the use of polyphosphate esters^{3,15}, TMSOTf⁶, RuCl₃¹⁷, SbCl₃¹⁸, ZnCl₂¹⁹, In(OTf)₃²⁰, I₂²¹, TEBA (benzyltriethylammonium chloride)²², silica/sulfuric acid²³, K₅CoW₁₂O₄₀·3H₂O²⁴ and heteropoly acids (H₃PW₁₂O₄₀)²⁵ as promoters for this Biginelli reaction. While in the original Biginelli protocol EtOH was used as protic solvent^{10,13,23}, suitable alternative solvents are MeCN^{16,18,20–21,25} and tetrahydrofuran (THF)¹⁴. With respect to environment-friendly procedures, several groups have developed solvent-free synthesis of monastrol^{3,15,17,19,22,24}.

In addition to the synthesis of racemic monastrol, some groups have isolated enantiomerically pure monastrol. The *S*-enantiomer has been proven to be the biologically more active enantiomer.²⁶ Enantioseparation of racemic monastrol was carried out by semi-preparative, enantioselective high-performance liquid chromatography (HPLC) by Kappe *et al.*, whereas the group of Dondoni reported on the chiral resolution through diastereomeric *N*-3 glycosyl amides to give the *R*- and *S*-enantiomer on a preparative scale¹⁴. For more information on the preparative HPLC enantioseparation of racemic monastrol via the *O*-*t*-butyldimethylsilyl derivative and subsequent rederivatization, we refer to the work of Cavazzini *et al.*²⁷. The first successful synthesis of *R*-monastrol with 99% ee via an asymmetric Biginelli reaction was recently developed by Zhu and co-workers, applying a chiral ytterbium catalyst²⁸.

MATERIALS

REAGENTS

- 3-Hydroxybenzaldehyde (Sigma-Aldrich, cat. no. H190808)
- Ethyl acetoacetate (Acros, cat. no. 1179700)
- Thiourea (Acros, cat. no. 138915)
- Ytterbium(III) trifluoromethanesulfonate hydrate (Yb(OTf)₃; Sigma-Aldrich, cat. no. 405329)
- Acetonitrile (MeCN; HPLC gradient grade; Fisher Scientific, cat. no. A/0627/17)
- Acetone (for analysis; Acros, cat. no. 1768000)
- Chloroform (CHCl₃; VWR, cat. no. 22706.361)
- Silica gel 60 (0.040–0.063 mm; Merck, cat. no. 1.09385)
- Sand (–50 + 70 mesh, Sigma-Aldrich, cat. no. 274739)

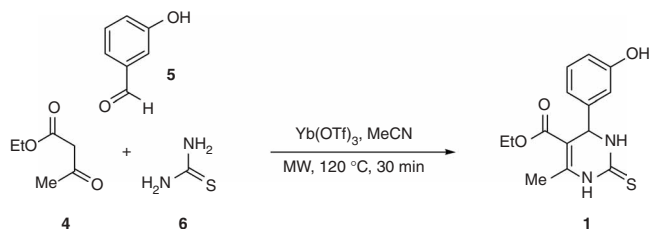


Figure 3 | Biginelli synthesis of monastrol.



Figure 2 | Initiator eight single-mode microwave reactor from Biotage AB (Uppsala).

The reaction conditions presented in this protocol are specifically optimized for the preparation of monastrol. Similar reaction and purification conditions can be applied for other DHPM derivatives^{29,30}. For the preparation of differently functionalized monastrol analogs, we think our protocol would be a good starting point and would deliver most of the desired derivatives in acceptable yields and purity. For more sensitive substrates, for example, furan-2-carbaldehydes, or special building-block combinations, a lower temperature and/or a prolonged reaction time might be required to obtain high yields^{13,31}. For some of the derivatives, the purification issue can be even simplified when the resulting DHPM products precipitate from the reaction mixture upon cooling and only a subsequent filtration step is necessary. If no precipitate is formed, the purification should be conducted as it is described in the procedure below.

- TLC aluminium sheets (silica gel 60 F₂₅₄; Merck, cat. No. 1.05554)
- EQUIPMENT**
- Microwave process vial (0.5–2 ml; Biotage)
- Magnetic stir bar (1 cm; Biotage)
- Micropipettes and tips (Finnpipette; Thermo)
- Teflon septum (Biotage)
- Aluminum crimp (Biotage)
- Microwave reactor (Initiator Eight; Biotage; see Fig. 2)
- Erlenmeyer flask
- Disposable glass pipettes
- Büchner funnel
- Filter paper (Macherey-Nagel)
- Round-bottom flask (10 ml)
- Chromatographic column (1.5 cm i.d. × 51 cm length)
- Test tubes (10 ml)
- HPLC with dual wavelength UV detector (Shimadzu LC-10)
- HPLC column: LiChrospher 100, C18 reversed phase, 100 × 3 mm, particle size 5 μm (E. Merck, cat. no. 151232)

EQUIPMENT SETUP

HPLC The UV detector is set at 215 and 303 nm. The separations were carried out at 25 °C using a mobile phase from (A) 0.1% TFA in 90:10 water/MeCN and (B) 0.1% TFA in MeCN (all solvents were HPLC grade, Acros; TFA was analytical reagent grade, Aldrich). The following gradients were applied at a flow rate of 0.5 ml min⁻¹: linear increase from 30% solution B to 100% solution B in 8 min, hold at 100% solution B for 2 min.

PROCEDURE

- 1| Place a stir bar in the microwave vial.
- 2| Add 61 mg (0.5 mmol) 3-hydroxybenzaldehyde, 96 μ l (0.75 mmol, 1.5 equivalent) ethyl acetoacetate (20–200 μ l Finnpi-
ette), 38 mg (0.5 mmol) thiourea, 31 mg (10 mol%) Yb(OTf)₃ and 0.5 ml MeCN (100–1000 μ l Finnpi-
ette) to the microwave vial.
- 3| Fit the Teflon septum into the aluminum crimp and crimp the vial.
▲ **CRITICAL STEP** The cap on the microwave vial must be even and tight; otherwise leakage of the reagents or solvent can occur under microwave irradiation.
- 4| Put the microwave vial in the proper position in the rack of the microwave instrument.
- 5| Program the microwave reactor (see table below).

Time	30 min
Temperature	120 °C
Pre-stirring	15 sec
Vial type	0.5–2 ml
Absorption level	Normal
Fixed hold time	On

! **CAUTION** The solvent is heated well above its boiling point, so all necessary precautions should be taken when performing such experiments. Vessels designed to withstand elevated temperatures must be used. After completion of an experiment, the vessel must be allowed to cool to a temperature below the boiling point of the solvent before removal from the cavity and opening to the atmosphere.

- 6| After cooling to 50 °C via gas jet cooling (compressed air, 5 bar), remove the vial from the rack and open it. Purify the product by either precipitation and filtration (A) or column chromatography (B).

(A) Precipitation and filtration

- (i) Fill an Erlenmeyer flask with crushed ice (approximately 10 g).
- (ii) Transfer the reaction mixture from the vial to the Erlenmeyer flask filled with crushed ice using a glass pipette.
- (iii) Rinse the microwave vial with an additional 250 μ l MeCN, and transfer it with a glass pipette to the Erlenmeyer flask filled with crushed ice.
- (iv) Let the reaction mixture stir vigorously for 1 h on a magnetic stirrer.
■ **PAUSE POINT** Can be left overnight.
- (v) Filter the light yellow precipitate through a Büchner funnel and wash it with 4 ml of cold water.

(B) Column chromatography

- (i) Transfer the reaction mixture from the vial into a round bottom flask with a glass pipette.
- (ii) Rinse the vial with an additional 0.5 ml of MeCN.
- (iii) Evaporate the solvent using a rotavap.
- (iv) Pack a chromatography column with ~10 g of silica gel using CHCl₃/acetone 5:1.
- (v) Dissolve the reaction mixture in ~0.5 ml of CHCl₃/acetone 5:1, load it onto the column and cover the top of the column with a layer of sand.
- (vi) Elute the column under gravity using CHCl₃/acetone 5:1 (approximate flow rate: 1 ml min⁻¹).
- (vii) After running through ~20 ml of eluent, start to collect ~5 ml fractions.

BOX 1 | ADVANTAGES OF MICROWAVE SYNTHESIS

- Higher reaction temperatures can be obtained by combining rapid microwave heating with sealed-vessel (autoclave) technology.
- In many instances significantly reduced reaction times, higher yields and cleaner reaction profiles will be experienced, allowing for more rapid reaction optimization and library synthesis.
- Lower boiling solvents can be used under pressure (closed-vessel conditions) and be heated at temperatures considerably higher than their boiling point.
- Microwave heating allows direct ‘in core’ heating of the reaction mixture, which results in a faster and more even heating of the reaction mixture.
- Easy online control of temperature and pressure profiles is possible, which leads to more reproducible reaction conditions.
- Can easily be adapted to automated sequential synthesis.

PROTOCOL

- (viii) Identify fractions containing the product by TLC using CHCl₃/acetone 5:1 as solvent mixture (fractions 4–9; R_f: 0.42)
(ix) Combine the fractions containing product and evaporate the solvent.
(x) Either dry the product using a vacuum pump or go further to Step 7.

7| Dry the product in the drying oven at 50 °C overnight.

8| Check the purity by HPLC using the HPLC-method described in HPLC setup.

? TROUBLESHOOTING

● TIMING

Steps 1–4: 5 min

Steps 5–6: 32 min

Steps 6A(i–iv): 62 min

Step 6A(v): 10 min

Steps 6B(i–iii): 10 min

Steps 6B(iv–vii): 1h 15 min

Steps 6B(viii–x): 2–3 h

Step 7: 15 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reasons	Solution
Isolated product not pure (according to HPLC at 215 nm)	Not washed properly during filtration using method A	Prepare a cold EtOH/H ₂ O 1:2 mixture for the washing step during filtration or recrystallize the product from EtOH
	Fractions do not contain pure product using method B	Use more silica gel, vary the eluting solvent mixture or triturate the isolated product with a small amount of diethyl ether
Lower yields	Temperature is lower than indicated	Check if the IR-sensor of the microwave instrument is calibrated correctly
	Too much solvent used for the washing step	If EtOH/H ₂ O 1:2 is used for the washing, reduce the amount or extract the mother liquor with ethyl acetate
	Leakage of the vial	Ensure that the vial is capped properly otherwise solvent can evaporate during the reaction and the temperature measurement is not correct anymore

ANTICIPATED RESULTS

The typical isolated yield of monastrol is 76% applying purification method A and 86% with method B. A >99% purity according to HPLC at 215 nm (retention time: 3.80 min) was obtained employing both purification protocols A and B.
mp 184–186 °C

¹H-NMR (360 MHz, DMSO-*d*₆) δ 1.12 (t, *J* = 7.1 Hz, 3H), 2.27 (s, 3H), 4.02 (q, *J* = 7.1, 2H), 5.08 (d, *J* = 3.4 Hz, 1H), 6.64 (d, *J* = 7.9 Hz, 3H), 7.11 (t, *J* = 7.6 Hz, 1H), 9.43 (s, 1H), 9.60 (s, 1H), 10.29 (s, 1H)

¹³C-NMR (DMSO-*d*₆) δ 14.0, 17.2, 54.0, 59.6, 100.8, 113.3, 114.6, 117.0, 129.5, 144.8, 144.9, 157.5, 165.2, 174.2.

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