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X-Ray Structure, Conformational Analysis, Enantioseparation, and Determination of Absolute Configuration of the Mitotic Kinesin Eg5 Inhibitor Monastrol☆

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Abstract—The conformational features of the mitotic kinesin Eg5 inhibitor monastrol were investigated by computational (AM1, HF/3-21G*), X-ray diffraction, and NMR studies showing that monastrol is a conformationally highly flexible molecule. Racemic monastrol was resolved by direct enantioselective HPLC and the absolute configuration of the first eluting (*S*)-(+) enantiomer was established by CD spectroscopy.

Introduction

A common strategy for cancer therapy is the development of drugs that interrupt the cell cycle during the stage of mitosis. Compounds that perturb microtubule shortening (depolymerization) or lengthening (polymerization) cause arrest of the cell cycle in mitosis due to perturbation of the normal microtubule dynamics necessary for chromosome movement. A variety of such drugs that bind to tubulin and thus inhibit spindle assembly are currently used in cancer therapy (e.g paclitaxel, docetaxel).[1]

By screening a 16,320-member library of diverse small molecules, Mayer et al.[2] have recently identified a novel cell-permeable molecule that blocks normal bipolar mitotic spindle assembly in mammalian cells and therefore causes cell cycle arrest. By combining several screening assays it was established that the compound monastrol (**1**) blocks mitosis by specifically inhibiting the motor activity of the mitotic kinesin Eg5, a motor protein required for spindle bipolarity.[2] Monastrol is the only cell-permeable molecule currently known to specifically inhibit mitotic kinesin Eg5 and can therefore be considered as a lead for the development of new anticancer drugs.[2]

A prerequisite for any understanding of the interaction of monastrol with mitotic kinesin Eg5 at the molecular level is the knowledge of the molecular geometry and probable bioactive conformation of monastrol itself. Here, we report a detailed structural characterization of monastrol (**1**). The geometry and conformational features of **1** were evaluated by X-ray crystallography, NMR spectroscopy, and computational methods. To address possible enantioselective effects in the molecular activity we have resolved racemic monastrol and established the absolute configuration of individual enantiomers by CD spectroscopy.

Results and Discussion

Monastrol (**1**) belongs to a family of dihydropyrimidine heterocycles (so-called DHPMs) that have been known for more than 100 years.[3] At present several general methods for the preparation of such DHPMs are feasible, including various solid-phase modifications suitable for combinatorial chemistry.[4] However, the detailed synthesis[2] and properties of monastrol (**1**) itself have not been reported in the literature. We have prepared racemic monastrol (**1**) by microwave-promoted condensation of ethyl acetoacetate, 3-hydroxybenzaldehyde, and thiourea, in a solventless variation of the classical Biginelli dihydropyrimidine synthesis using polyphosphate ester (PPE) as reaction medium (Scheme 1).[5] This procedure provides monastrol in ca. 60% yield and high purity after chromatographic work-up. In our hands the traditional ethanol/HCl three-component condensation protocol (reflux, 3 h)[2,6] gave a much lower yield (17%).

☆ Synthesis and reactions of Biginelli compounds, part 19; part 18 see: Saloutin, V. I.; Burgart, Ya. V.; Kuzueva, O. G.; Kappe, C. O.; Chupakhin, O. N. *J. Fluorine Chem.* **2000**, *103*,17.

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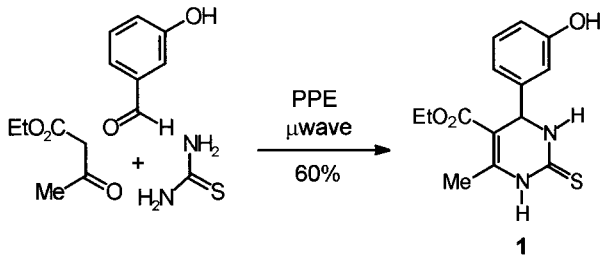

Scheme 1. Synthesis of monastrol (**1**) by microwave-promoted (μwave) three-component Biginelli condensation.

DHPMs of the monastrol type are known to be conformationally flexible molecules, in which the aryl rings and the ester groups can rotate and the conformation of the dihydropyrimidine ring can change.[7–9] We have therefore carried out semiempirical (AM1) and ab initio (HF/3-21G*) geometry optimizations of the possible monastrol conformers. Four distinct local minima[7] were found for geometries where (a) the ester group is in coplanar arrangement with the double bond of the dihydropyrimidine ring (carbonyl group *cis* or *trans* with respect to the C5═C6 double bond), and (b) where the hydroxy substituent on the C4-aryl ring adopts either a syn- (*sp*) or antiperiplanar (*ap*) orientation with respect to C4-H. In all these conformers the aryl ring is positioned axially, perpendicular to, and (nearly) bisecting the half-boat-like dihydropyrimidine ring (no minima were found for equatorially arranged C4-aryl rings).[7,8] For both computational methods the lowest energy conformation was predicted to be the one where the ester group was oriented *cis* and the aryl group had *sp* conformation (*cis*/*sp*) (Fig. 1). However, the three other conformers (*cis*/*ap*, *trans*/*sp*, *trans*/*ap*) had energies that were only 0.25–0.97 kcal/mol (AM1) or 0.66–1.85 kcal/mol (HF/3-21G*) above the lowest energy *cis*/*sp* conformer.

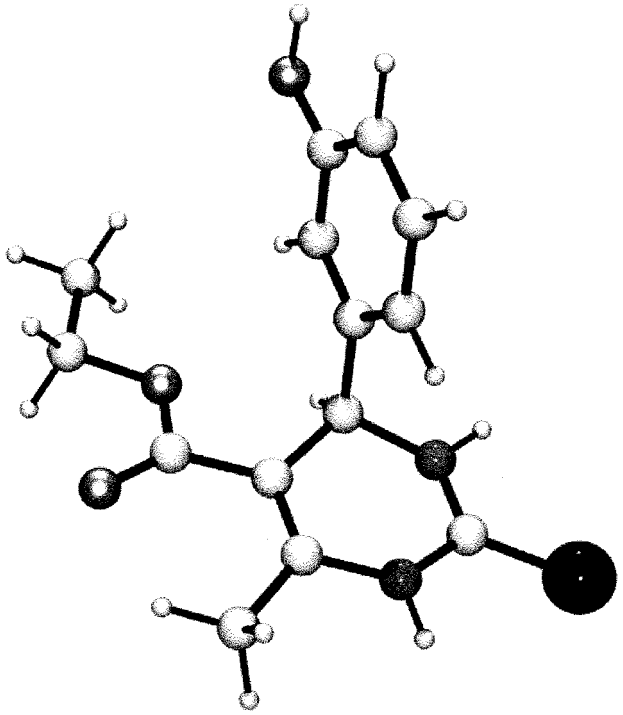

Figure 1. HF/3-21G* optimized geometry of the lowest energy conformer (*cis*/*sp*) of monastrol.

The structure and geometry of **1** in the solid state was established by a single crystal X-ray structure analysis (Fig. 2). The half-boat-like conformation of the dihydropyrimidine ring with the aryl ring in an axial position (perpendicular to and bisecting the heterocyclic ring) is clearly evident. The exocyclic ester at C5 has *trans* orientation with respect to the C5═C6 double bond, and the hydroxy substituent on the C4-aryl ring adopts the antiperiplanar (*ap*) position. This *trans*/*ap* conformation found in the solid-state corresponds to a geometry that is somewhat higher in energy, according to both AM1 (0.77 kcal/mol) and HF/3-21G* (1.85 kcal/mol) calculations, than the calculated minimum energy *cis*/*sp* conformation shown in Fig. 1. The observed solid-state conformation is likely to be a result of the crystal packing forces. The crystal packing diagram (not shown) shows intermolecular H-bonding between the *trans* carbonyl oxygen and the N1-H atom of the neighboring molecule, apparently overriding the small calculated preference (0.5–1.0 kcal/mol) for *cis* ester orientation.

Monastrol (**1**) therefore shows a high degree of conformational flexibility. Based on previous calculations for related systems the rotational barriers for ester group and aryl ring rotation in monastrol can be expected to be relatively small.[7–9] In solution or in a biological environment all four calculated minimum energy conformations therefore seem accessible. In order to validate this experimentally we have carried out NMR experiments in solution. The conformational flexibility of the aryl moiety was established by different NOE measurements (360 MHz, benzene-d_6, 25°C): irradiation of the C4-H signal at 5.36 ppm (d, J=3.46 Hz), led to a significant NOE enhancement for both aromatic ortho protons, i.e. the C2′-H at 7.33 ppm (dd, J=2.04 and 1.60 Hz, 5% NOE) and the C6′-H at 7.02 ppm (ddd, J=6.83, 1.60 and 1.60 Hz, 4% NOE). This indicates that in solution both the *sp*- and *ap*-aryl conformers are present, in agreement with the low calculated energy differences and barriers for these rotamers (see above). The conformational orientation of the C5 ester group in **1** is more difficult to ascertain and seems strongly dependent on solvent interactions. While in some polar solvents (DMSO-d_6, DMF-d_7) one distinct quartet (J=7.5 Hz) for the OCH_2 group at ca. 4.0 ppm was observed, in other solvents (acetone-d_6, $CDBr_3$, benzene-d_6) more complex splitting patterns were found at room temperature, indicating solvent interactions and/or hindered rotation. Intermolecular interactions, i.e. H-bonding of the ester carbonyl group with a suitable donor (i.e. solvent), may preferentially stabilize the *cis* or *trans* conformer, as also seen in solid state structures where in a series of closely related DHPMs either *cis*, *trans* or both conformers can be found.[7–9] It is therefore reasonable to assume that in a biological environment both conformers are readily accessible with no predictable preference for either rotamer.

Since the inhibition of mitotic kinesin Eg5 may well be dependent on the absolute configuration at the C4 stereocenter of monastrol, we have carried out an enantioseparation of racemic monastrol and characterized the individual enantiomers. In order to obtain enantiomerically pure

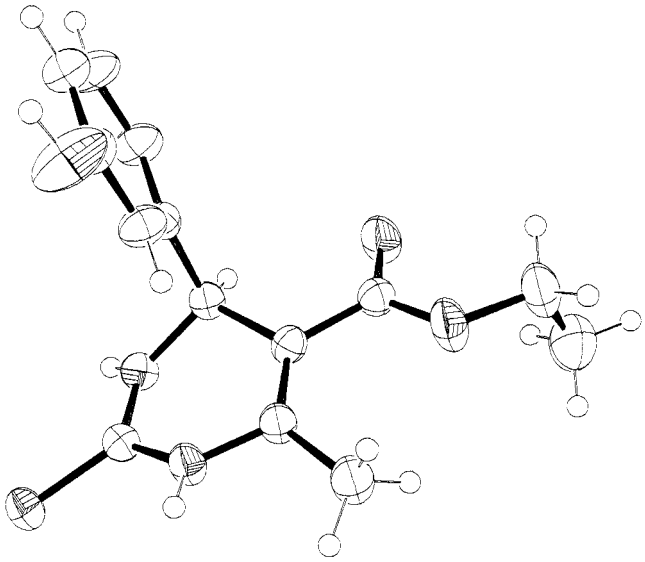

Figure 2. Solid-state structure of monastrol (**1**).

samples of monastrol for comparative CD measurements racemic **1** was resolved by direct HPLC enantioseparation using a Chiralcel OD-H column.[10] This was done by simple collection of the eluting enantiomers from a semi preparative-type HPLC separation (see Experimental). After the mobile phase was evaporated, both enantiomers (>99% ee) were dissolved in methanol and their CD spectra and optical rotation measured. The CD spectra of the first (—) and second (—) eluting enantiomer of **1** are shown in Fig. 3. The spectrum of the first eluting enantiomer displays a positive Cotton effect at 303 nm and a negative effect around 232 nm, whereas the second eluting enantiomer exhibits the corresponding mirror-image CD. The corresponding UV spectrum (not shown) exhibits a strong absorption maximum at 306 nm, ϵ=13,200. Based on a comparison with reference CD spectra of DHPMs of known absolute configuration[11] the first eluting enantiomer showing a positive Cotton effect at 303 nm was assigned the (*S*)-configuration. Note that it is the characteristic CD activity of the enamide chromophor of the heterocyclic dihydropyrimidine skeleton at ca. 300 nm that allows the assignment of absolute configuration in this series of DHPM analogs.[11] Also note that the observed elution order of the monastrol enantiomers on the cellulose-derived Chiralcel OD-H column agrees nicely with previous DHPM enantioseparations on this chiral stationary phase.[11] The optical rotation was determined to be: (*S*)-**1**: $[\alpha]_{436}$=+1.1 (*c*=0.007, methanol).

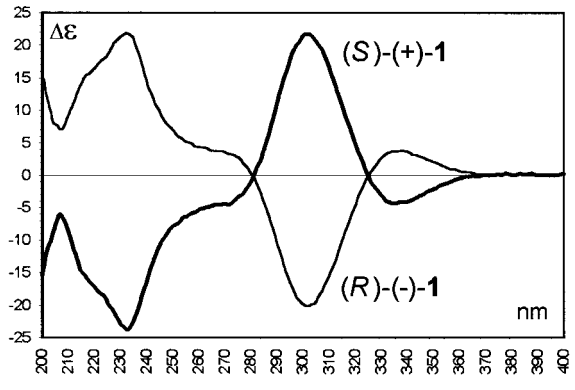

Figure 3. CD spectra of (*S*)- and (*R*)-monastrol (**1**) in methanol.

In conclusion, we have demonstrated that the mitotic kinesin Eg5 inhibitor monastrol is a conformationally mobile heterocyclic system. Based on the evaluation of calculated (AM1, HF/3-21G*) and experimental (X-ray, NMR) conformer structures it can be concluded that in a biological environment four distinct minimum geometries are accessible, with no clear preference for one particular conformer. The successful chiral resolution of monastrol will provide the opportunity to study the inhibitory effect of individual enantiomers on mitotic kinesin Eg5.

Experimental

Melting points were determined on a Gallenkamp melting point apparatus Mod. MFB-595 and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained on a Varian XL-200 Gemini instrument at 200 MHz and 50 MHz, respectively. Solvent-dependent ^{1}H NMR experiments and different NOE effects for **1** were measured on a Bruker AMX 360 instrument at 360 MHz. IR spectra were recorded on a Perkin–Elmer 298 spectrophotometer. Micro-analyses were obtained on a Fisons Mod. EA 1108 elemental analyzer.

Ethyl 6-methyl-4-(3-hydroxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (1). A mixture of ethyl acetoacetate (300 mg, 2.3 mmol), 3-hydroxybenzaldehyde (244 mg, 2.0 mmol), thiourea (380 mg, 5 mmol), and polyphosphate ester (PPE, 300 mg) was placed in a 20 mL glass beaker. After the mixture was stirred for 10–20 s with a spatula the reaction container was inserted into a 400 mL pyrex beaker filled with neutral alumina (150 g). This set-up was irradiated in a domestic microwave oven 5 times at full power (800 W) for 10 s each with 1–2 min cooling periods in between each irradiation cycle. EtOH (5 mL) was added to the hot reaction mixture, which was subsequently poured onto ice (50 g). The precipitated crude product was purified

by silica gel chromatography (hexane/EtOAc 2:1) to yield 350 mg (60%) of colorless product, mp 184–186°C (MeCN). ^{1}H NMR (200 MHz, DMSO-d_6): 1.14 (t, J=7.5 Hz, 3H), 2.30 (s, 3H), 4.03 (q, J=7.5 Hz, 2H), 5.11 (d, J=3.5 Hz. 1H), 6.61–6.69 (m, 3H), 7.06–7.17 (m, 1H), 9.45, 9.62, 10.31 (3 brs); ^{13}C NMR (DMSO-d_6): 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; IR (KBr) 3300, 3180, 2900–2600, 1670, 1655, 1620, 1575 cm^{-1}; calcd for $C_{14}H_{16}N_2O_3S$: C, 57.52; H, 5.52; N, 9.58. Found: C, 57.33; H, 5.52; N, 9.34.

Computational methods

Semiempirical AM1 calculations were carried out using the PC Spartan Pro package (Version 1.0.1)[12] on a Pentium PC. Starting geometries were obtained using Spartans interactive building mode, and preoptimized using the SYBYL force field. Starting geometries for individual conformers were obtained by performing systematic bond rotations around the C6-phenyl and C5-ester single bonds in order to ensure that the global minimum for each conformer had been located. Ab initio calculations were carried out at the HF/3-21G(*) level of theory with the PC Spartan Pro package. Geometries were completely optimized and convergence was achieved in all optimizations.

X-Ray structure determination

Colorless crystals (grown from ethanol-hexane mixture), $C_{14}H_{15}N_2O_3S$ (291.34), triclinic (P$\bar{1}$); a=7.231(2) Å, b=8.794(3) Å, c=12.016(3) Å, α(=77.33(2), β=82.13(2), γ(=77.99(2), V=725.8(4) Å^3, Z=2. The structure determination was performed on an automatic four-circle Siemens P3/PC diffractometer, using graphite monochromatized MoK_α radiation (0.71069 Å) at 293 K, $\theta/2\theta$ scanning, $2\theta_{max}$=50°. The structure was solved by direct methods based on 2455 unique reflections (1894 significant reflections with $F>4\sigma(F)$) using the SHELX97 package. The final value of conventional R-factor R1 was 0.046 (184 parameters, refinement against F^2). Crystallographic data for compound **1** have been deposited at the Cambridge Crystallographic Data Center (CCDC).

HPLC separation

High performance liquid chromatographical measurements employed a Hewlett Packard HP 1050 compact system with variable wavelength detector (VWL) and a HP ^{2D}HPLC Chemstation version A.02.05. The chiral stationary phase used for the direct enantioseparation of racemic monastrol **1** was a Chiralcel OD-H column (J.T. Baker, Netherlands) (250×4.6 mm i.d., 5 μm). The temperature during the separation was adjusted to 25°C. The flow rate was set to 1.0 mL/min and UV detection performed at 254 nm. For the semi-preparative separation a solution of ca. 0.02 mg of racemate in 50 μL of mobile phase (*n*-heptane/2-propanol 90:10) was subjected to HPLC separation (α=1.31, R_s=1.78). After evaporation of the mobile phase at 40°C the collected individual enantiomers were dissolved in ca. 400 μL methanol for CD measurements. The optical rotation was measured on a Perkin–Elmer Polarimeter using a 10 cm Microcell employing the pooled (*S*)-enantiomers of 10 semipreparative runs (MeOH).

Circular dichroism spectroscopy

Measurements were carried out on a JASCO J-715 CD spectropolarimeter at 20°C using a 1 mm quartz cell with a volume of 350 μL. The following instrument settings were used: band width 1.0 nm, resolution 1 nm, accumulation 2, sensitivity 20 mdeg, response 1 s, speed 100 nm/min.

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