Anti-Cancer Drugs

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Conformational Studies and Atropisomerism Kinetics of the ALK Clinical Candidate Lorlatinib (PF-06463922) and Desmethyl Congeners

Jeff Elleraas, Jason Ewanicki, Ted W. Johnson, Neal W. Sach, Michael R. Collins, and Paul F. Richardson**

Abstract: *Lorlatinib (PF-06463922) is an ALK/ROS1 inhibitor and is in clinical trials for the treatment of ALK positive or ROS1 positive NSCLC (i.e. specific subsets of NSCLC). One of the laboratory objectives for this molecule indicated that it would be desirable to advance a molecule which was CNS penetrant in order to treat brain metastases. From this perspective, a macrocyclic template was attractive for a number of reasons. In particular, this template reduces the number of rotatable bonds, provides the potential to shield polar surface area and reinforces binding through a restricted conformation. All of these features led to better permeability for the molecules of interest and thus increased the chance for better blood brain barrier penetration. With a CNS penetrant molecule, kinase selectivity is a key consideration particularly with regard to proteins such as TrkB, which are believed to influence cognitive function. Removal of the chiral benzylic methyl substituent from lorlatinib was perceived as not only a means to simplify synthetic complexity, but also as a strategy to further truncate the molecule of interest. Examination of the NMR of the desmethyl analogues revealed that the compound existed as a mixture of atropisomers, which proved separable by chiral SFC. The individual atropisomers were evaluated through a series of in vitro assays, and shown to have a favorable selectivity profile when compared to lorlatinib. The challenge to develop such a molecule lies in the rate at which the atropisomers interchange dictated by the energy barrier required to do this. Here, we describe the synthesis of the desmethyl macrocycles, conformational studies on the atropisomers, and the kinetics of the interconversion. In addition, the corresponding conformational studies on lorlatinib are reported providing a hypothesis for why a single diastereomer is observed when the chiral benzylic methyl group is introduced.*

In drug discovery, it is well established that the separate enantiomers of a chiral molecule can differ significantly in properties such as biological activity, toxicity and metabolism,[1] and several high profile examples have served to emphasize these differences.[2] Evaluation of the properties of the individual enantiomers has long been positioned as a critical step in the development process, which in turn has led to the trend in which the vast majority of marketed compounds are currently single enantiomers.[3] More recently, the phenomenon of atropisomerism has been recognized as becoming more prevalent in the discovery and development of new molecular entities.[4] The hypothesis for this shift has been attributed to the relationship between the design of more compact and conformationally constrained inhibitors with the availability of new effective synthetic methods for the construction of the sterically hindered bonds contained within these molecules.[5] However, atropisomerism brings an additional layer of complexity to the development process.[6] Unlike enantiomers, which are usually relatively stable, and racemize via a bond-breaking and making process, atropisomers interconvert through an intramolecular dynamic process that in the majority of cases involves bond rotation. Given that bond rotation is time-dependent, the racemization half-lives for atropisomers vary from seconds to years depending on numerous factors including steric hindrance, temperature, electronics, solvent, etc. Furthermore, since the biological properties of the molecule may differ between the atropisomers,[7] it is critical to determine the racemization half-life (preferably under physiological conditions) in order to follow the best strategy for how to progress a molecule (single atropisomer, or mixture) through a program's development cascade. Alternatively, a series of design strategies have been proposed to modify a molecular structure to a related analogue, while possessing the desired activity against the biological target and mitigating the issue of atropisomerism.[8] Herein, we will demonstrate one such strategy in which the introduction of a stable stereogenic center prevents the inter-conversion of atropisomers leading to a single desired conformation being observed for the molecule of interest.

In addition to the potential complication of developing an atropisomeric compound, a further obstacle is the initial detection of atropisomers, and there are cases in which a molecule has been developed as a racemic mixture of atropisomers, the existence of which were only later revealed through chiral detection experiments.[9] With the growing knowledge base, atropisomerism within a lead molecule can be anticipated through the instinctive recognition of structural motifs, which lead to hindered axial rotation, or through computational predictions of the likelihood of a molecule presenting atropisomerism.[10] In the cases in which less

[*] J. Elleraas, J. Ewanicki, Dr. T. W. Johnson, N. W. Sach, M. R. Collins, Dr. P. F. Richardson
Oncology Medicinal Chemistry, Pfizer, La Jolla
10770 Science Center Drive, San Diego, CA 92121 (USA)
E-mail: michael.collins@pfizer.com
paul.f.richardson@pfizer.com

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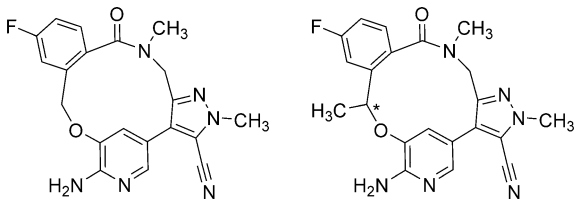
intuitive molecular fragments leading to conformational-based chirality are present, numerous analytical tools (NMR, X-ray, chiral HPLC) or even biological assays have been used to determine the existence of atropisomerism.[7,11]

The evaluation of macrocyclic structures as potential drug candidates is currently enjoying a significant renaissance.[12,13] In particular, these structural motifs are attractive with the perspective of being able to constrain the compound into the binding conformation, and pick up additional protein–ligand interactions. As part of our next generation anaplastic lymphoma kinase (ALK) inhibitor project[14] for the treatment of non-small cell lung cancer (NSCLC), we sought to develop a small molecule inhibitor that combined brain availability with broad-spectrum ALK potency for the treatment of patients who exhibit tumor progression while on crizotinib,[15] overcoming resistance mutations[16] and brain metastases.[17] This initiative led to the design and discovery of the macrocyclic inhibitor, lorlatinib (**2**.[18] In addition, the macrocyclic framework enables the number of rotatable bonds to be reduced leading to a more compact structure with increased buried surface area compared to the corresponding acyclic analogues, which we postulated would provide permeability advantages. In this initial disclosure, we highlighted a matched-pair analysis between a pair of macrocyclic inhibitors with and without the chiral methyl substituent at the C-10 position (Figure 1). At that time, we also made the qualitative observation regarding the desmethyl compound existing as a pair of atropisomers, which can be separated by chiral supercritical fluid chromatography (SFC), though interconversion occurs on standing in solution over several weeks. Herein, we describe the synthesis of the direct desmethyl analogue of our lead compound, lorlatinib, and provide a comparison of the biological activities of the pair of atropisomers as well as an in-depth analysis of the racemization kinetics, energetics, and the mechanism.

The inter-conversion of atropisomers within macrocycles and medium-sized rings takes place not through a single-bond rotation, but instead a "ring flip" occurs to transform one enantiomer into the other.[19] The rate of this inter-conversion is again dependent on a variety of factors most notably steric hindrance and ring size. Numerous examples of atropisomerism exist in medium-size rings and macrocycles. For example, telenzepine[20] represents a marketed compound in which the atropisomers are stable,



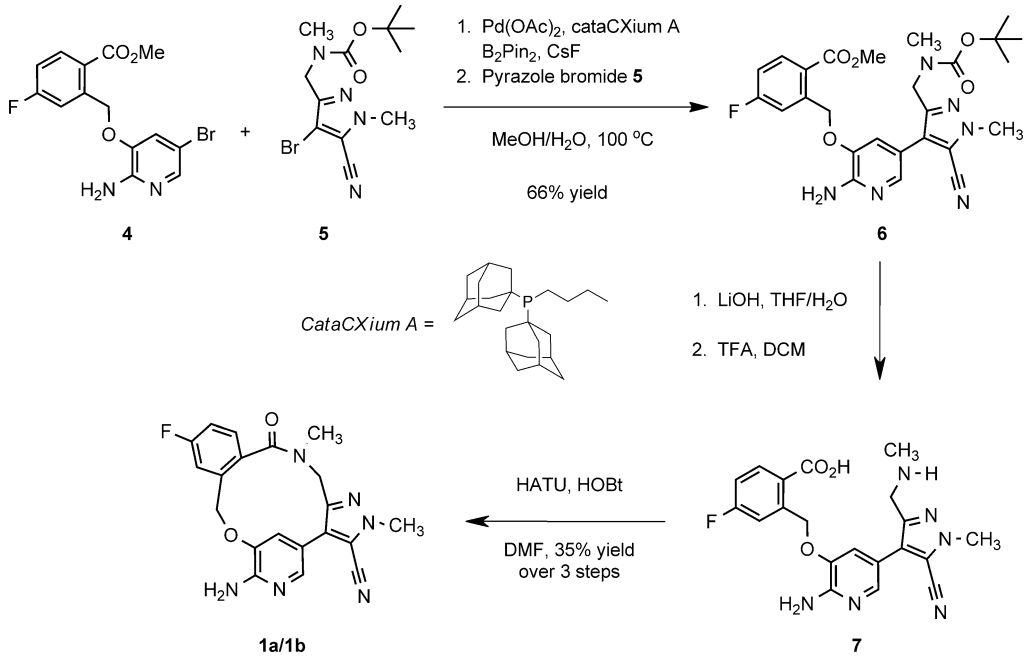
1a
1b

2, (*R*)-lorlatinib
3, (*S*)-lorlatinib

Figure 1. 12-membered macrocyclic ALK inhibitors differing by methyl substitution.

whereas nevirapine racemizes rapidly at room temperature and as such is developed as a single entity.[21] As noted, the strategy employed herein to progress the lead compound involved introduction of a second chiral center into the molecule, which brings about a favorable shift in the conformational equilibrium.

Scheme 1 highlights the synthesis of the desmethyl macrocycle **1a**/**1b**. The synthesis of the Boc-protected amino ester **6** utilized a Suzuki reaction where aryl bromide **4** was refluxed in an aqueous methanolic suspension in the presence of palladium acetate, cataCXium A, cesium fluoride and diboron pinacol ester. The pyrazole bromide **5** was subsequently added to the crude reaction mixture and refluxed overnight. Conversion of the Boc-protected amino methyl ester **6** to the amino acid **7** was accomplished by hydrolysis of the methyl ester followed by an acid mediated removal of the Boc protecting group. Lactamization of the amino acid **7** to the racemic atropisomer **1a**/**1b** was accomplished by slowly adding a solution of the amino acid **7** and DIEA in DMF to a solution of HATU and HOBt in DMF.



Scheme 1. Synthesis of racemic atropisomer **1a**/**1b**. TFA = trifluoroacetic acid, DCM = dichloromethane, HATU = *O*-(7-azabenzotriazol-1-yl)-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole.

Often the first sign of existence of atropisomers is provided by the NMR, and this was the case for compound **1a**/**1b**. ^{1}H NMR reveals the presence of enantiotopic protons as shown in Figure 2. For lorlatinib (**2**), the flipped conformers are diasteromeric, and the presence of a single conformer is confirmed by the presence of a single set of diastereotopic protons.

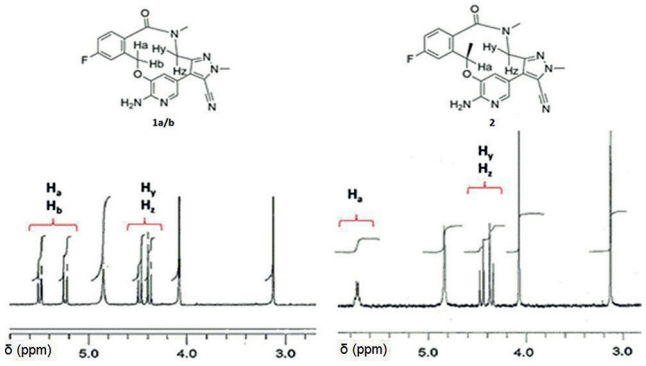


Figure 2. ^{1}H NMR comparison of compound **1a**/**1b** and **2** (PF-06463922).

With this information in hand, we were able to investigate the separation of compounds **1a** and **1b** using chiral SFC.[22] This enabled the individual isomers to be evaluated separately in a series of biological assays as described in Table 1.

Compounds **1a, 1b**, **2** and **3** were submitted to an in vitro panel assessing ALK-L1196M potency, CNS (central nervous system) exposure, ADME, and selectivity (Table 1). Compound **1a** had comparable biochemical and cell potency to **2**, while **1b** appears to be slightly less active. Although **1a** and **1b** were separated by chiral chromatography, it is likely that significant racemization occurred prior to and/or during testing, providing values more consistent with racemic mixtures. Indeed, modeling of **1b** is completely inconsistent with optimal binding and would be expected to provide significant losses in potency relative to **1a**, certainly more than the modest decrease observed (Figure 3, top panel). Compound **3**, the enantiomer of **2**, highlights the significance of the methyl chirality, losing several hundred fold in biochemical potency relative to the more active enantiomer **2**. The desmethyl analogues were slightly less lipophilic (log D 1.9) than the methyl analogue (log D 2.3), in line with expectations. All compounds showed low in vitro clearance in human liver microsomes. In addition, the compounds were highly permeable and showed low efflux ratios consistent with Pgp substrate incompatibility and increased potential for CNS exposure. Kinase and general selectivity were paramount given the required high tissue distribution of these analogues. TrkB was used as a selectivity surrogate based on its high ATP binding site sequence homology to ALK and its putative role in memory and cognition. In addition, achieving selectivity over TrkB was deemed to be critical given the potential adverse CNS-related effects due to TrkB inhibition.[23] The increased selectivity ratios for **1a** (831-fold) and **1b** (244-fold) relative to **2** (33-fold) were initially intriguing and prompted further investigation.

Table 1: Potency, ADME and selectivity of macrocyclic analogues.

Compound	ALK-L1196M K_i [nM]	pALK-L1196M cell IC_{50} [nM]	Log D[a]	HLM $Cl_{int,app}$[b]	MDR BA/AB (ratio)[c]	TrkB K_i [nM] (selectivity)[d]
1a	1.0	30	1.9	< 8	27.0/13.7 (2.0)	831 (831×)
1b	2.0	131	1.9	< 8	33.0/13.4 (2.5)	488 (244×)
2 (lorlatinib, PF-06463922)	0.70	21	2.3	< 8	28.0/19.3 (1.5)	23 (33×)
3	273	ND	2.2	< 8	23.5/12.1 (1.9)	ND

[a] Log D was measured at pH 7.4. [b] $Cl_{int,app}$ refers to the total intrinsic clearance obtained from scaling in vitro half-lives in human liver microsomes (HLM). [c] MDCK-MDR1 BA/AB efflux at 2 μM substrate concentration and pH 7.4. [d] TrkB K_i and ratio relative to ALK-L1196M K_i.

Although the atropisomers **1a** and **1b** are enantiomeric and energetically redundant (Figure 3, top panel), the transition barrier from one conformation to the other determines racemization rates and impacts time-dependent analytical methods. The 12-membered ring allows a small space for the inversion, but several atoms must pass through the inside of the ring in order to arrive at each distinct conformation. This passage of atoms through a tight space gives rise to the higher energy barrier and atropisomerism.

The addition of a methyl group to form compound **2**/**2′** changes the conformational dynamics drastically. Figure 3 (bottom panel) highlights two distinct conformers of methyl analogue **2**/**2′**. The benzylic center in **2** minimizes A-1,3 strain while **2′** maximizes A-1,3 strain.[24] Hypothetically, even if the inversion barrier were low enough to allow access to both conformers, the large energetic ground state difference ($+8.5$ kcal mol^{-1}) would drive the equilibrium completely to the bioactive conformer **2**. However, this is not the probable mechanistic scenario which more likely involves the late transition state during the cyclization step driving the product to the desired structure **2** where it is unable to access the inverted conformation due to high energetic penalties.[25]

One of the most common methods for determining activation energies involves determining the coalescence temperature of two NMR resonances of the interconverting species.[26] Variable temperature NMR was carried out on Compound **1a** (see Supporting Information). As can be seen, no coalescence was observed up to 110 °C, and as such an alternative method was utilized for examining the activation energy through an Eyring plot.[27]

We then envisioned that we would employ the segmented flow technology for the measurement of the racemization kinetics.[28] However, despite the advantages of this methodology with both the automation and the unprecedented temperature control offered by the Accendo Conjure,[29] there are drawbacks particularly given the introduction of a disconnect between the emergence/processing of segments from the instrument and the actual chiral analysis. Although, for molecules with a high barrier to inter-conversion (such as (*S*)-

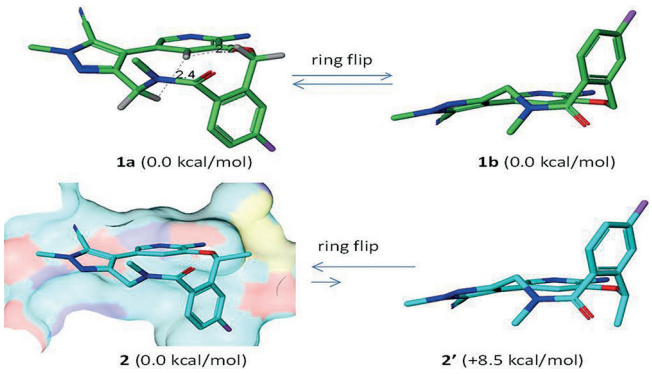


Figure 3. Top panel: Enantiomeric low-energy conformations of **1a** and **1b**. Bottom panel: Low-energy bioactive conformation of methyl analogue **2** with ALK-L1196M KD surface (PDB 4CLJ). Diastereomeric ring-flipped, locally minimized conformation (**2′**) with additional strain of 8.5 kcal mol^{-1} relative to locally minimized conformation **2.** Compounds were locally minimized and energetic differences calculated using the OPLS2001 force field.

BINOL), the potential errors from this disconnect are practically non-existent and this method is unique in offering the ability to accelerate the kinetics through heating solvents above the boiling point. For molecules though in which $t_{1/2}$ is in the range of hours, delays in sample analyses and background epimerization of stock solutions can introduce a degree of error. The first step in carrying out our analyses was to determine which measurements to determine our kinetic analyses at, and how best to conduct these experiments to minimize the potential errors.

Our initial range finding experiments at 25, 80, 110 and 140 °C confirmed this hypothesis, and indicated that we would need an alternative method for collecting data on the kinetics of the atropisomerization. Although, the 25 °C sample after 1 h showed 98 % *ee*, all three of the other samples had racemized completely. Switching to 25, 40 and 55 °C, and evaluating after 24 h intervals indicated that the 55 °C sample was racemic after 24 h. However, the 25 and 40 °C samples showed *ee* values of 84.2 % and 27.9 % respectively. Allowing these samples to stir at temperature for a further 24 hours lead to values of 70 % (25 °C) and 12.4 % (40 °C), whereas after 72 hours, the 40 °C sample had racemized whilst the 25 °C sample showed an *ee* of 34 %. This gave us a temperature range to work within as well as indicating that regular in-line sampling would be optimal in order to obtain the most accurate data.

The iChem Explorer is ideally suited for measuring the kinetics of the atropisomerization as this instrument adds heating and stirring capabilities to an HPLC instrument enabling chemistry to be carried out with automated in-line sampling. The instrument is compatible with a range of Agilent HPLC instruments, and enables heating up to 150 °C with stirring rates of up to 1200 rpm using a magnetic stirrer bar placed in a vial.[30] Typically, a 2 mL vial is used to evaluate the chemistry making this a relatively material-sparring method for the evaluation of the kinetics. In contrast to the segmented-flow method previously described, only one aliquot of material needs to be prepared for each temperature evaluated with the instrument capable of taking samples at pre-determined intervals. In addition, to eliminate initial drift after sample preparation, the iChem Explorer is capable of dispensing the required volume of solvent (in this case IPA (isopropyl alcohol)) to the solid material at the outset of the experiment. From our range finding studies, we decided to evaluate 25, 45 and 65 °C with automated sampling at 5 minute intervals. The in-line HPLC was set up with a variation of the chiral method developed for SFC in order to determine the *ee* at each time point, and the data able to be evaluated in real time. Sampling was continued so that the reactions were monitored for at least two to three half-lives to ensure that the inter-conversion approximates to a first-order reaction. From these experiments, the rate constant of racemization (k_{rac}) can be determined at each of the temperatures evaluated by plotting ln (% *ee*) vs. *t* in which the slope provides the rate. The half-life can then be determined from the equation $t_{1/2} = \ln 2/k_{rac}$. Examples of the iChem data, and the kinetics plot for the 65 °C run is provided in Figure 4 with Table 2 providing the data for the three temperatures evaluated.

Table 2: Measured first-order rate constants for the racemization of compound **1a** in IPA.

Entry	*T* [°C]	k_{rac} [min^{-1}]	R^2	$t_{1/2}$ [min]
1	25	0.0002	0.9674	3466
2	45	0.0096	0.9926	72.2
3	65	0.1356	0.9966	5.11

Figure 5 shows the Eyring plot derived from the experimental kinetic data. Using the three points, the Eyring equation calculates $\Delta H^{\ddagger} = 32.1$ kcal mol^{-1} and $\Delta S^{\ddagger} = 0.0243$ kcal mol^{-1} K. These parameters provide a calculated Gibbs energy of activation $\Delta G^{\ddagger}$ to be 24.6 kcal mol^{-1} at physiological temperature (37 °C), which corresponds to a $t_{1/2}$ of approximately 6 hours in this medium.[31]

With this data in hand, it was also important to establish the stability of the macrocyclic compound, lorlatinib (**2**) and determine the chiral integrity. An analogous experiment was set up on the iChem Explorer heating a sample of lorlatinib (spiked with the enantiomer **3** to ensure both compounds are detected in the chiral assay). After 24 hours at 65 °C in IPA, no chiral erosion of the sample material was detected (see Supporting Information).[32]

During our search for broad-spectrum, brain-penetrant ALK inhibitors, we discovered, initially by H NMR, that some 12-membered macrocycles displayed atropisomerism. These analogues displayed excellent in vitro potency and ADME properties, but were not progressed due to the complications associated with atropisomerism. In addition, a unique use of iChem Explorer with in-line sampling allowed us to define half-lives and transition state barrier energies for interconversion of the atropisomers. The chiral benzylic methyl of lorlatinib rigidifies the 12-membered template and does not display complicating conformational effects. Calculations confirmed these observations based on ground-state energies. Lorlatinib also shows excellent in vitro and

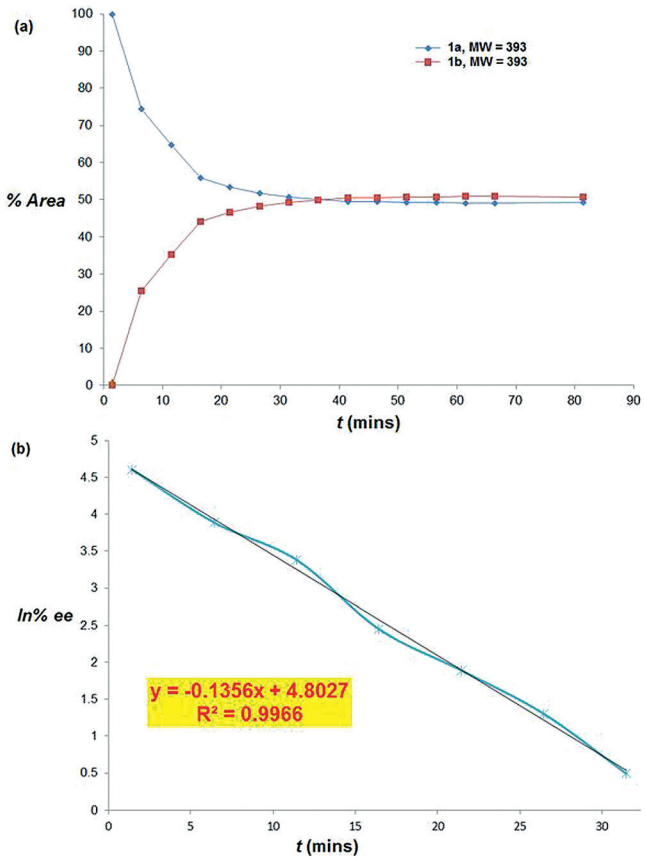


Figure 4. a) Representative iChem Explorer sampling data and b) first-order kinetics plot for the racemization of compound **1a** in IPA at 65 °C.

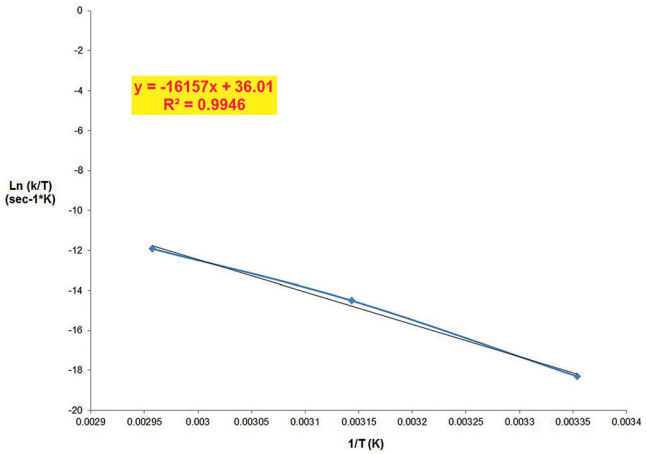


Figure 5. Eyring plot for the atropisomerization of compound **1a** in IPA.

in vivo properties, and is currently in Phase I/II clinical trials.[33]

Acknowledgements

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Keywords: atropisomers · conformational analysis · EML4-ALK · macrocycles · racemization

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