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Heeb, J-P., Clayden, J., Smith, M., & Armstrong, R. J. (2023). Interrogating the Configurational Stability of Atropisomers. *Nature Protocols*. https://doi.org/10.1038/s41596-023-00859-y

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Interrogating the Configurational Stability of Atropisomers

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Abstract: Atropisomers are molecules whose stereogenicity arises from restricted rotation about a single bond and are of current importance due to their applications in catalysis, medicine, and materials science. The defining feature of atropisomeric molecules is that their stereoisomers may interconvert by bond rotation: as a result, evaluating their configurational stability (i.e., the rate at which their stereoisomers interconvert) is central to any work in this area. Important atropisometric scaffolds include C–C linked biaryls (such as the ligand BINAP and the drug vancomycin) and C–N linked amine derivatives such as the drug telenzepine. This article focusses upon the three most widely used experimental methods that are available to measure the rate of racemization in atropisomers, namely: (i) kinetic analysis of the racemization of an enantioenriched sample: (ii) dynamic HPLC; and (iii) variable temperature NMR. For each technique, an explanation of the theory is set out, followed by a detailed experimental procedure. A discussion is also included of which technique to try when confronted with a new molecular structure whose properties are not yet known. None of the three procedures require complex experimental techniques, and all can be performed using standard analytical equipment (NMR and HPLC). The time taken to determine a racemization rate depends on which experimental method is required, but for a new compound it is generally possible to measure a racemization rate in <1 day.

BACKGROUND:

Atropisomers are molecules in which rotation about a single bond is sufficiently slow that different conformers can be isolated as separate chemical species.[1] The archetypal examples

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are axially chiral biaryls such as BINAP,[2] which have been widely studied due to their privileged role as ligands for asymmetric catalysis,[3,4] but atropisomerism is also possible in many other molecular scaffolds, including axially chiral heterobiaryls,[5,6] anilides,[7-9] aromatic carboxamides,[10] and diaryl amines (Fig. 1a).[11,12] In addition to applications in catalysis, atropisomeric molecules have also been widely employed in materials science and are increasingly recognised to be important for healthcare, with an estimated 15% of small molecule pharmaceuticals containing at least one axis of chirality.[13] These important applications have driven the development of a variety of elegant stereoselective approaches to the synthesis of atropisomeric molecules. Illustrated for a generic biaryl **A** in Fig. 1b, these include: (i) asymmetric cross-coupling; (ii) strategic ring formation; (iii) ring opening of strained intermediates; and (iv) functionalization of existing scaffolds via desymmetrization, kinetic resolution or dynamic kinetic resolution.[4]

However, unlike conventional chiral molecules containing stereogenic centres, the enantiomers of atropisomeric molecules can be interconverted by bond rotation. For example, **A** can be transformed into its enantiomer *ent*-**A** by rotation about the biaryl axis (Fig. 1b). If this process is rapid, an enantioenriched sample will rapidly decay to a racemic mixture, effectively precluding any single enantiomer applications. Consequently, determining the configurational stability of atropisomeric molecules (i.e., the rate at which an enantiopure sample undergoes racemization) is central for any study in this area. This is the focus of this article, which summarizes the three most widely used techniques, in each case providing an explanation of the theory followed by a detailed experimental procedure illustrated with examples from our own laboratories. This is the first article in which the various experimental procedures for the determination of atropisomeric stability are comprehensively described. The reader is also directed to an excellent review article by Mayor and co-workers detailing the physical and mathematical principles involved in such measurements.[14]

Enantiomerisation versus Racemisation

Before embarking upon a detailed discussion, it is first necessary to define some key terminology, beginning with the rather subtle difference between "enantiomerization" and "racemization".[15] A generalised scenario is presented in Fig. 1c involving a generic atropisomer whose enantiomers (R) and (S) can be interconverted. Enantiomerization refers to the microscopic process in which a pair of enantiomers are reversibly exchanged. The first-order rate constant for enantiomerization is defined as k_{ent}, and it is important to note that the rate constant for the reverse process (k_{ent}^{-1}) must have an identical magnitude.

In contrast, racemization refers to a macroscopic process in which a bulk enantioenriched sample is irreversibly converted to racemic material (or material with lower enantiomeric excess). Each individual enantiomerization event transforms one (R)-configured molecule to one with (S)-configuration, and hence the enantiomeric excess is reduced by two molecules. *This means that the rate of racemization is double that of enantiomerization*, and the magnitude of the rate constant for racemization (k_{rac}) is therefore double that of k_{ent} (box 1, eq. i).

The racemization rate constant is usually expressed as a more tangible racemization half-life ($t^{1/2}_{rac}$), which can straightforwardly be calculated using eq. ii, and represents the time taken for an enantiopure sample to reach 50% e.e. The Eyring equation (box 1, eq. iii) also enables a value for k_{ent} to be used to determine the Gibbs activation energy of enantiomerization ($\Delta G^{\ddagger}$), which is another convenient means to report configurational stability. As for any first order chemical process, k_{ent}, k_{rac} and $t^{1/2}_{rac}$ display a strong dependence upon temperature. However, it is important to recognise that $\Delta G^{\ddagger}$ is also a temperature-dependent parameter ($\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$).

The temperature dependence of $\Delta G^{\ddagger}$ is rather more subtle, and as we shall see it is common practice to determine a high-temperature value of $\Delta G^{\ddagger}$ and, assuming it to be temperature invariant, estimate a value for k_{rac} and $t^{1/2}_{rac}$ at room temperature. This is a useful approach,

but it is important to recognise that it is an approximation and will be inaccurate in cases where the contribution of entropy to $\Delta G^{\ddagger}$ is more important.

Conformational Isomers Versus Atropisomers

As set out in Fig. 2, the rate of bond rotation can vary widely, from rapid exchange of conformers (low $t^{1/2}_{rac}$, low $\Delta G^{\ddagger}$) to atropisomers with very high configurational stability (high $t^{1/2}_{rac}$, high $\Delta G^{\ddagger}$). Therefore, the point at which a molecule is considered atropisomeric is arbitrary and, in fact, two different definitions are available. The first was introduced by Oki in 1983, and states that for a molecule to be defined as atropisomeric its conformers must interconvert with $t^{1/2}_{rac} > 1000$ s (~17 min) at a given temperature.[1] Alternatively, the more recent classification system introduced by LaPlante looks at a continuum from conformational isomers to atropisomers,[16] placing molecules into three different categories:

(i)   class 1 atropisomers, which have $\Delta G^{\ddagger} < 20$ kcal/mol at 25 °C and are effectively conformationally unstable, with racemization half-lives under one minute;

(ii)   class 2 atropisomers, which display intermediate conformational stability with $\Delta G^{\ddagger}$ values in the range 20-28 kcal/mol at 25 °C. Working with enantioenriched class two atropisomers is possible, but is challenging owing to their relatively rapid rate of racemization;

(iii)   class 3 atropisomers, which have $\Delta G^{\ddagger} > 28$ kcal/mol, and can generally be considered to be configurationally robust molecules that can be handled and manipulated without risk of racemization (unless high temperatures are required).

When a new (suspected) atropisomeric molecule is prepared, it is very important to determine its stability to racemization – i.e., where on the scale in Fig. 2 it falls. However, this is often challenging to predict in advance, especially if the scaffold concerned is a novel one. Computational analysis can be very useful in this context to provide approximate racemization barriers but becomes increasingly complicated in systems with multiple rotatable bonds.

Therefore, for most studies involving new atropisomeric molecules, it is necessary to determine racemization rates experimentally. The focus of this article is upon the techniques that are available to carry out such measurements. Many experimental approaches are possible, but the three most widely used procedures are:

(1) kinetic analysis of the racemization of an enantioenriched sample;

(2) dynamic HPLC; and

(3) variable temperature NMR.

For each procedure, a brief summary of the theory is presented, followed by step-by-step instructions explaining how the technique can be performed illustrated by worked examples. Some background to each of these approaches is summarized below followed by a detailed experimental procedure for each illustrated with worked examples. An additional technique, exchange spectroscopy (EXSY), is suitable for timescales in between those of dynamic HPLC and VT NMR, but is not covered by this article.[17]

Procedure 1: Kinetic Analysis of the Racemization of an Enantioenriched Sample:

Arguably the most intuitive way to determine the configurational stability of an atropisomer is to prepare an enantioenriched sample (for example by preparative HPLC separation of a racemic sample) and then over a period of time, carry out several analytical measurements of its enantiomeric ratio to assess how quickly racemization occurs (Fig. 3a).

Racemization is a first-order kinetic process, and therefore the enantiomeric excess is expected to decrease exponentially over time, from enantiopurity (e.e. = 1) tending towards racemic product (e.e. = 0) as shown in Fig 3b. The racemization half-life ($t^{1/2}_{rac}$) corresponds to the time taken for the enantiomeric excess to decrease to 50% of its optically pure form ($e.e._{t=0}$). The rate of exponential decay is given by eq. iv, but it is practically more useful to rearrange this to eq. v, which tells us that a plot of ln(1/e.e.) vs time should be a straight line with a gradient of

k_{rac} (Fig. 3c). It is particularly important to note that the starting sample does not have to be fully enantiopure to perform this type of analysis – in this case, $e.e._{t=0} \neq 1$ and the graph will have a non-zero y-intercept, but the gradient still represents k_{rac}.

To illustrate this method, we have chosen a specific example, namely the axially chiral enol ether **1**, whose preparation *via* cation-directed *O*-alkylation of tetralones (Fig. 4) we recently reported.[18] In this case, analysis was achieved by high performance liquid chromatography (HPLC) on a chiral stationary phase. HPLC is the most commonly used analytical technique for this procedure, but it is important to note that the same concept and mathematical treatment can also be applied to other analytical methods (e.g., supercritical fluid chromatography and gas chromatography). The crucial requirement for this method is that the enantiopure compound possesses sufficient configurationally stability to enable several measurements of its e.e. to be taken before it becomes too low to measure. In practice, this usually requires $t^{1/2}_{rac}$ to be greater than ~20 min at room temperature, but a good rule of thumb is that the method will likely be suitable if analytical HPLC of the racemic compound on a chiral stationary phase provides two peaks with baseline separation. Depending on the conformational stability, racemization can be monitored at room temperature or at elevated temperature.

Procedure 2: Dynamic HPLC

As shown schematically in Fig. 5a, analysis of a racemic atropisomer by HPLC on a chiral stationary phase yields three possible outcomes depending upon its configurational stability:

(i)   If $t^{1/2}_{rac}$ is significantly shorter than the difference in retention times, then averaging will lead to a single peak.

(ii)   If $t^{1/2}_{rac}$ is comparable to the difference in retention times, then an unusual peak shape will be observed consisting of two peaks flanking a distinctive non-baseline plateau or bulge;

(iii) $t^{1/2}{}_{rac}$ is significantly longer than the difference in retention times, then two baseline-separated peaks will be observed.

We have already dealt with the latter scenario (Procedure 1), but Procedure 2 involves determining configurational stability in scenario (ii) when evidence of dynamic behaviour is observed by HPLC analysis. The unusual plateauing observed in these spectra is colloquially known as "the Batman effect" and it occurs because the two enantiomers interconvert as they pass through the HPLC column. Based only on the line shape of such a peak, it is possible to calculate the rate constant for enantiomerization using the unified equation developed by Trapp and co-workers (Fig. 5b). [19-23]

However, the equation is complex, requiring parametrisation of the peak in a number of ways (e.g., measuring the peak heights, the height of the plateau, the width of each peak at half-height and the retention time of the peaks). Fortunately, we do not need to worry about any of the detail of this calculation, because the Trapp group have also designed the powerful, freely available software package [DCXplorer,[24] which can analyse raw HPLC data for 'Batman'-type chromatograms, directly providing a value for k_{ent}.

Procedure 2 provides a detailed explanation of how racemization rates can be calculated using this method, illustrated with a specific example of a class 1 atropisomer, namely racemic diarylamine *rac*-**2** (Fig. 6). We recently reported that this compound (and analogues) display an unusual form of atropisomerism, in which enantiomers (e.g., **2** and *ent*-**2**) are possible due to slow rotation about the sterically encumbered Ar–NH–Ar motif.[11]

Procedure 3: Variable Temperature NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most powerful tools available for the study and characterization of small molecules, relying upon the idea that each nuclear environment in a given molecule generates a different signal, providing information about its

chemical environment and proximity to other nuclei. Variable temperature NMR (VT-NMR) is used to study scenarios in which two nuclei in inequivalent environments (either in the same molecule or another) undergo exchange. This is particularly important in atropisomeric molecules – for example, consider a generic axially chiral biaryl **3**, which can be interconverted with its enantiomer *ent*-**3** by bond rotation (Fig. 7a).

The introduction of a stereogenic axis will lead to diastereotopicity in any prochiral groups, for example Me_a and Me_b are chemically inequivalent, and may display different chemical shifts in the ^1H NMR spectrum. The two enantiomers **3** and *ent*-**3** must of course have identical NMR spectra, but the enantiomerization process exchanges the chemical environment of the diastereotopic groups Me_a and Me_b, and as a result also exchanges their NMR signals. The critical question is how the rate of the interconversion (k_{ent}) compares to the NMR timescale. Depending on the rate of the process, there are three possible scenarios (Fig. 7b):

(i)   enantiomerization is fast compared to the ^1H NMR timescale – in this case, we see averaging of the two signals to produce a single sharp peak;

(ii)   enantiomerization occurs at a comparable rate to the ^1H NMR timescale resulting in a broad peak (or peaks);

(iii)   enantiomerization is slow compared to the ^1H NMR timescale – in this case, we observe the methyl groups as two well defined, separate peaks.

It is worth noting at this point that the following methods can be applied universally to any NMR active nuclei undergoing exchange, however, ^1H VT-NMR is the most widely used due to the ubiquitous nature of ^1H nuclei and their favorable NMR properties. This method has been widely used to determine racemization rates, [11,25-27] but it is important to note that it can be applied only to molecules containing diastereotopic groups, e.g., benzyl (R–**CH₂**–Ar), ethyl (R–**CH₂**–Me) and isopropyl groups (R–C**Me₂**–H).

The enantiomerization rate is governed by temperature (faster at high temperature, and slower at low temperature), and the key idea of a VT-NMR experiment is to record NMR spectra at different temperatures and thereby identify the temperature at which separate signals coalesce, that is, where the trough between the two peaks has just reached the same intensity as the peaks themselves (known as the coalescence temperature, T_c). We can use the following equation (eq. vi) to calculate the enantiomerization rate constant (k_{ent}) at the coalescence temperature for uncoupled systems, i.e., singlets, (where Δv is the frequency difference between the two signals when exchange is slow):[28]

$$\text{(eq. vi)} \qquad k_{ent} \text{ at } T_c = \frac{\pi \, \Delta v}{\sqrt{2}}$$

However, when considering mutually exchanging AB systems, the following, slightly modified equation (eq. vii) must be used instead to account for the coupling:

$$\text{(eq. vii)} \qquad k_{ent} \text{ at } T_c = \pi \sqrt{\frac{\Delta v^2 + 6J_{AA'}^2}{2}} \approx 2.22 \sqrt{\Delta v^2 + 6J_{AA'}^2}$$

Armed with both values for T_c and k_{ent} at this temperature, we can then estimate $\Delta G^{\ddagger}$ at T_c, using the Eyring equation. This classical VT-NMR method has been widely used to determine k_{ent} and $\Delta G^{\ddagger}$ at T_c, however, these formulae cannot be used to predict these variables for other temperatures unless the magnetic field strength is also changed. It is important to note that chemical shifts may also be temperature dependent possibly leading to inaccurate values for k_{ent}. While more precise methods for calculating rate constants are available (*vide infra*), the error associated with these equations was reviewed by Raban et al. and found to be small, for interconverting enantiomers, except for strongly 'roof-topped' signals where Δv is small relative to J.[29]

Perhaps the most precise technique for estimating rate constants at different temperatures is line shape analysis (LSA), where the rate of enantiomerization can be directly calculated from NMR spectra recorded around the coalescence temperature. This significant improvement in accuracy and quantity of data comes at the cost of time and complexity. Different software packages are available for LSA of VT-NMR spectra. Some of the most established include SpinWorks,[30] gNMR,[31] or TopSpin's DNMR module.[32] In this article we shall focus on the dynamic NMR module freely available in TopSpin 3. Both methods are best illustrated with a worked example, and procedure 3 below sets out a detailed method to measure the enantiomerization rate of diarylamine *rac*-**4** (Fig. 8).[11]

An important consideration: With high/low temperature NMR experiments, the temperature sensor of the probe does not necessarily reflect the precise internal temperature of the sample; indeed, it may deviate by several degrees.[33] Running VT-NMR experiments with uncalibrated temperatures will usually give a reasonable approximation of k_{ent} (this is how the data presented below for **4** was collected). However, if more accuracy is required, an internal or external NMR thermometer must be used to determine the sample temperature. Full details of this method are beyond the scope of this article but have been described previously in the literature.[34,35] The accurate temperature values measured can be used in lieu of the approximate values in the analysis below.

Selecting the most appropriate procedure:

In our experience, between these three procedures it should be possible to determine racemization rates for the majority of atropisomeric compounds. Procedure 1 is suitable for molecules which undergo slow racemization ($\Delta G^{\ddagger} \geq 95$ kJ/mol), Procedure 2 for molecules which undergo racemization on an intermediate timescale ($\Delta G^{\ddagger} \approx 80\text{-}95$ kJ/mol), and Procedure 3 for molecules in which racemization is relatively rapid ($\Delta G^{\ddagger} \leq 85$ kJ/mol, assuming a 10 Hz peak separation and the ability to collect data up to 400 K). For many

compounds there will already be evidence in the literature which provides an indication of the likely rate of racemization and which method will be most appropriate. However, in some cases it will be necessary to decide which procedure to use when confronted with a new material whose properties are completely unknown. A typical workplan for such a situation is provided in Fig. 9.

The first step is to carry out analytical HPLC on a chiral stationary phase. If a baseline separated pair of enantiomers, or 'Batman'-shaped chromatogram is observed, the answer is simple and Procedure 1 or 2 respectively should be used. If a single broad peak is observed, it is possible that this could be due to atropisomerism on the timescale of the HPLC experiment. This can be tested by reanalysing the sample by HPLC with the chiral column cooled to a lower temperature (e.g., 5 °C). If a 'Batman' profile is observed then Procedure 2 can be used, if not, it is likely broadening is occurring for some other reason and the sample should be treated as a single peak. Formation of a single peak could be a consequence of fast bond rotation producing an averaged signal, or it could simply reflect poor resolution of enantiomers. Therefore, the sample should be run on all HPLC columns available. If in all cases a single peak is observed, then it is increasingly probable that racemization is occurring too rapidly to be observed by HPLC and Procedure 3 should be applied.

MATERIALS:

REAGENTS

! CAUTION Solvents and reagents are potentially harmful. Apply the appropriate safety procedures. Wear gloves, a laboratory coat and safety glasses. Dispose appropriately of all chemicals used.

Solvents for HPLC (Procedure 1 and 2)

<CRITICAL> These are the two solvents most commonly employed for normal phase HPLC in our laboratories. Many alternatives are possible, but care should be taken to ensure compatibility with the chiral column as each chiral stationary phase tolerates a different range of solvents

- Hexane suitable for HPLC (e.g., Sigma-Aldrich, cat. No. 34859). **! CAUTION** Inhalation may damage the nervous system. Handle only inside a fume cupboard.
- 2-Propanol suitable for HPLC (e.g., Sigma-Aldrich, cat. no. 1010402500).

Solvent for Procedure 1 Reflux

- *m*-Xylene (e.g., Sigma-Aldrich, cat. no. 296325)

  <CRITICAL> This was chosen, because the high boiling point allows a sample to be kept in solution at a constant high temperature. Other inert solvents with even higher boiling points (for example 1,2-dichlorobenzene) may be used for racemizations at temperatures >140 °C.

A selection of deuterated NMR solvents for room temperature or low temperature analysis (Procedure 3)

- Chloroform-*d* (e.g., Sigma-Aldrich, cat. no. 151823). **m.p. = –64 °C. b.p. = 60.9 °C.**

  ! CAUTION Toxic if inhaled, suspected of causing cancer and damaging the unborn child. Handle only inside a fume cupboard.

- Benzene-*d₆* (e.g., Sigma-Aldrich, cat. no. 151815). **m.p. = 6.8 °C. b.p. = 79.1 °C.**

  ! CAUTION May cause cancer. May cause genetic damage as well as damage to organs. Wear gloves and handle only inside a fume cupboard.

- Methanol-*d₄* (e.g., Sigma-Aldrich, cat. no. 151947). **m.p. = –99 °C. b.p. = 65.4 °C.**

  ! CAUTION Toxic if swallowed, in contact with skin or if inhaled. Causes damage to organs. Wear gloves and handle only inside a fume cupboard.

- D_2O (e.g., Sigma-Aldrich, cat. no. 435767). **m.p. = 4 °C. b.p. = 101.4 °C.**

A selection of deuterated NMR solvents for high temperature analysis (Procedure 3)

<CRITICAL> *Many more solvents can be used, including even non-deuterated alternatives, however, it is important that the signals of interest (i.e., exchanging nuclei) can be clearly distinguished and integrated; and this is usually precluded either by solvent peaks or impurities.*

- Toluene-*d₈* (e.g., Sigma-Aldrich, cat. no. 434388). **m.p. = -84 °C. b.p. = 110 °C.**

  ! CAUTION Inhalation may damage the nervous system. Handle only inside a fume cupboard.

- Dimethyl sulfoxide-*d₆*, (e.g., Sigma-Aldrich, cat. no. 151874). **m.p. = 20.2 °C. b.p. = 189 °C.**

- *p*-Xylene-*d₁₀*, (e.g., Sigma-Aldrich, cat. no. 175927). **m.p. = 13 °C. b.p. = 135 °C.**

- 1,1,2,2-Tetrachloroethane-d₂ (e.g., Sigma-Aldrich, cat. no. 358703). **m.p. = -44 °C. b.p. = 147 °C. ! CAUTION** Fatal in contact with skin or if inhaled. Wear gloves and handle only inside a fume cupboard.

EQUIPMENT

- 4 decimal place analytical balance (e.g., Ohaus PR224 or equivalent)

HPLC

- 2 mL Glass Screw Top Vials for analytical HPLC (e.g., ThermoFisher Scientific, cat. no. 6ASV9-1P or equivalent)

- 9 mm Screw Caps for HPLC vials (e.g., ThermoFisher Scientific, cat. No. 6ASC9RT1 or equivalent)

- Glass Inserts for 2 mL Vials with pulled point, total volume <0.4 mL (e.g., ThermoFisher Scientific, cat. no. 6PME04C1 or equivalent)

- An analytical HPLC system comprising: (i) a pump capable of delivering a flow rate of at least 1 mL/min; (ii) an autosampler or port for manual injection capable of injecting at least 100 μL; (iii) a UV detector e.g., DAD or VWD. *Optional:* (iv) a thermostatically controlled column compartment; (v) Fraction collector.

HPLC columns

<CRITICAL> For Procedures 1 and 2, you will need a selection of HPLC columns with a chiral stationary phase. In our experience, the suite of columns below can achieve enantiomeric separation for most compounds.

- Chiralpak AD-H 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 19325)

- Chiralcel OD-H 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 14325)

- Chiralpak IA 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 80325)

- Chiralpak IB-N 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 88325)

- Chiralpak IC 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 83325)

- Chiralpak IG 250 x 4.6 mm, 5 μm particle size (Daicel cat. no. 87325)

- *Optional (but recommended):* Associated guard columns for each column.

Reflux experiment in Procedure 1

- 25 mL three-necked round-bottomed flask

- Ground glass stopper

- Liebig condenser

- Quickfit screwthread thermometer adapter (e.g., Fisher Scientific, cat. no. 10187988)

- 6 mm Teflon coated stirring bar

- Stirrer hotplate with immersion temperature probe (e.g., IKA RCT basic or equivalent)

- Crystallization dish (~1 L capacity) containing ~800 mL of silicon oil and a 5 cm Teflon coated stirring bar.

- Glass thermometer suitable for use up to 200 °C

- Glass Pasteur pipettes (150 mm)

NMR experiments in Procedure 3:

- 1.75 mL Glass Screw Top Vial (e.g., Fisher Scientific, cat. no. 15374779 or equivalent).

- Syringe (e.g., 1 mL).

- Standard 5mm borosilicate NMR tubes with plastic caps.

- Glass Pasteur pipettes (150 mm).

- NMR spectrometer capable of obtaining ^{1}H-NMR at ≥ 400 MHz (i.e., > 9.4 T) over a range of temperatures (e.g., Bruker Avance III or equivalent).

PROCEDURE 1: Kinetic Analysis of the Racemization of an Enantioenriched Sample:

Stage 1: Optimizing an Analytical HPLC Method ●*Timing 1-3 h*

1. Weigh approx. 1 mg of the racemic atropisomeric compound (e.g., **1**) into a 2 mL glass HPLC vial.

2. Add 1.5 mL of Hexane and agitate gently. If the sample remains undissolved, add the minimum volume of 2-propanol required to achieve dissolution. Polar compounds can be dissolved in neat 2-propanol.

3. Carry out standard analytical HPLC runs (isocratic solvent composition; 1 mL/min; 5 µL injection volume) aiming to achieve baseline separation of the two enantiomer peaks. A full description of this step is beyond the scope of this article, but we have found the following general principles helpful when trying to separate the enantiomers of novel chiral compounds.

   ▪ Begin by selecting any chiral column and carry out isocratic runs with various ratios of hexane/2-propanol until a retention time of approximately 10 min is obtained.

   ▪ Using the same eluent ratio, evaluate all other chiral columns that are available and select the one that gives the best separation of enantiomers.

   ▪ If only partial separation is achieved in all of these runs, select the sample with the best separation and run again with a less polar eluant mixture.

   ▪ Sometimes separation can be improved by reducing the flow rate to 0.5 mL/min or chilling the column compartment (typically 5 °C).

   ▪ If adequate separation still cannot be obtained, then try another solvent system (e.g., heptane/*tert*-butyl methyl ether).

   After applying the steps above, compound **1** gave an analytical HPLC spectrum with baseline separation of enantiomers (Fig. 10).[18]

Δ **CRITICAL STEP** It is essential to achieve baseline separation of the two enantiomer peaks before progressing to the next step.

? TROUBLESHOOTING

Stage 2: Obtaining an Enantioenriched sample ●*Timing 30 min*

4. The simplest case is that in which enantioenriched material is already available. For example, with axially chiral enol ether **1**, enantioenriched material (96:4 e.r.) was available through asymmetric catalysis.[18] In this case, proceed directly to Step 10.

    If only racemic material is available, then it is necessary to separate a single enantiomer by semi-preparative HPLC. To do this, perform Steps 5-9.

5. Inspect the analytical HPLC data and make a note of the retention time range over which one enantiomer elutes, e.g., see Fig. 11a for a generic example, with a suitable target region highlighted in pink.

6. Now perform a semi-preparative HPLC run using the same 1 mg/mL sample prepared in steps 1-2. Use the same analytical chiral column and conditions optimised above, but use a larger injection volume e.g., 100 μL.

    (Note: a standard 250 x 4.6 mm analytical column is suitable for this small-scale separation, but a semi-preparative or preparative HPLC column can be used if available)

7. Collect the desired enantiomer peak (i.e., collect during the time interval specified in step 5). This can be achieved using a fraction collector, or manually by collecting the waste stream from the UV detector. If the manual method is to be used, it is advisable to attach ~20 cm of HPLC tubing to the detector outlet, collecting unwanted waste in a large vial, then switching to collect the desired fractions in a clean 2 mL HPLC vial (Fig. 11b).

(Note: Depending upon the specific HPLC software used, it may also be possible to monitor the data in real-time to work out when to start and stop collecting)

8. Repeat steps 6-7 several times and pool together the fractions containing enantioenriched material.

9. If a different solvent is required for the next stage, then concentrate the sample by placing it under a gentle stream of nitrogen for 10-15 mins.

   Λ CRITICAL STEP It is essential to achieve relatively high levels of enantioenrichment at this stage (>70:30 e.r.).

   ? TROUBLESHOOTING

Stage 3: Monitoring the Rate of Racemization

●*Timing highly variable – 2 hours to 2 weeks depending on sample.*

<CRITICAL> Steps 10-17 are followed if the study is performed at a temperature other than room temperature. In the specific example described, the temperature of the experiment is about 140 °C. If the racemization study is to be performed at room temperature, it is possible to avoid steps 10-17, by instead making up an analytical HPLC sample of enantioenriched material and directly analysing multiple injections from the HPLC vial. In this case, the ambient temperature should be recorded using a thermometer.

<CRITICAL> For a novel compound, the temperature and time interval between runs may require some optimization to ensure a measurable, but not excessive amount of racemization occurs. The ideal scenario is a change of 5-10% e.e. between each measurement. This procedure works equally well for molecules racemizing more slowly (aliquots can be removed over the course of days, weeks or months) and the method can be applied in an identical manner to assess the configurational stability of non-biaryl scaffolds including cyclic/acyclic and C-C, C-X, and X-X atropisomeric molecules.[7]

10. Weigh 5 mg of enantioenriched compound (e.g., **1**) into a 25 mL three necked flask and add a Teflon coated magnetic stirrer bar followed by 5 mL of *m*-Xylene.

    Note: It is also possible to use much lower concentrations of analyte, e.g., by dissolving microgram quantities obtained by semi-preparative HPLC separation, provided the sensitivity of the HPLC detector is great enough.

11. Fit the central neck with a jacketed Liebig condenser, and insert a thermometer adaptor into one side arm. Cap the final neck with a ground glass joint.

12. Insert a high temperature thermometer via the thermometer adaptor and immerse it into the solution inside the flask.

13. Immerse the flask into a preheated oil bath such that the flask is submerged up to the solvent level (Fig. 12a). For compound **1**, an oil temperature of 150 °C was used, which was sufficient to reflux the *m*-xylene solvent (b.p. = 139 °C).

14. Allow the flask to equilibrate for 5 minutes.

15. Note the internal temperature from the thermometer and record it. For compound **1**, an internal temperature of 139 °C (412 K) was observed.

    Δ CRITICAL STEP It is essential that the temperature of the oil bath remains stable (±1 °C) throughout the subsequent steps.

16. Quickly remove the glass stopper, and withdraw a small aliquot (~200 μL) using a Pasteur pipette and transfer into a clean HPLC vial containing a glass insert. Replace the glass stopper and accurately record the time at which the aliquot was removed – this represents $t = 0$.

17. Analyse the resulting sample by HPLC on a chiral stationary phase (5 μL injection volume).

    ? TROUBLESHOOTING

18. At regular (and accurately recorded) time intervals, remove further aliquots and perform HPLC analysis using identical conditions to those used in step 17. For compound **1**, a total

of seven analytical runs were performed over the course of 4 hours (raw HPLC data is shown in Fig. 12b).

19. For each sample, compare the aliquot time with that at which the initial sample was removed, and calculate a time (in seconds). Integrate the corresponding spectrum to determine the enantiomeric ratio and calculate ln(1/e.e.). For compound **1**, this led to the data shown in Fig. 12c.

20. Using Microsoft Excel, plot this data as a line graph with time (in seconds) on the x-axis and ln(1/e.e.) on the y-axis and fit a linear regression line. For compound **1**, the resulting graph is shown in Fig. 12d along with the equation and R^2 value.

21. Note the gradient of this line graph; this gradient corresponds to k_{rac}. For example, for compound **1** (the gradient of the graph in Figure 12d):

$$k_{rac} = 1.82 \times 10^{-4} \text{ s}^{-1} \text{ (at 139 °C)}$$

22. Substitute this into eq. ii to calculate the corresponding racemization half-life:

$$t_{rac}^{1/2} = \frac{ln2}{k_{rac}} = \frac{0.693}{1.82 \times 10^{-4}} = 3.81 \times 10^3 \text{ s} = 1.06 \text{ hours (at 139 °C)}$$

23. Once you have determined k_{rac}, calculate the rate constant for enantiomerization (see eq. i):

$$k_{ent} = \frac{k_{rac}}{2} = \frac{1.82 \times 10^{-4}}{2} = 9.10 \times 10^{-5} \text{ s}^{-1} \text{ (at 139 °C)}$$

24. Substitute this value of k_{ent} along with the temperature noted in step 15 (412.15 K) into the Eyring equation (eq. iii) to calculate the Gibbs activation energy ($\Delta G^{\ddagger}$). For example, for compound **1**:

$$\Delta G^{\ddagger} = RTln\left(\frac{k_B T}{k_{ent} h}\right) = 8.314 \times 412.15 \times ln\left(\frac{1.381 \times 10^{-23} \times 412.15}{9.10 \times 10^{-5} \times 6.626 \times 10^{-34}}\right)$$

$$= 1.34 \times 10^5 \text{ J/mol}$$

$$= 134 \text{ kJ/mol} = 32.0 \text{ kcal/mol}$$

25. As mentioned in the introduction, it is sometimes helpful to make the crude assumption that $\Delta G^{\ddagger}$ is invariant with temperature and thereby estimate the room temperature racemization half-life. For this type of calculation, we recommend the use of the freely available online "Eyring equation calculator".[36]

Using this online tool, enter a value for $\Delta G^{\ddagger}$ (in either kJ/mol or kcal/mol) and temperature (in K); the corresponding rate constant (k_{ent}) and enantiomerization half-life are displayed. The latter can simply be halved to obtain $t^{1/2}_{rac}$. For example, entering our calculated value of $\Delta G^{\ddagger}$ for compound **1** confirms a value for $t^{1/2}_{rac}$ of 1.06 hours at 412.25 K (Fig. 13a) and estimates a room temperature racemization half-life of approximately 500 years (Fig. 13b). As discussed above, it is important to remember that this is a very rough approximation, but it is nevertheless useful, allowing us to conclude that no appreciable racemization of **1** is likely to occur at room temperature. For samples analysed at room temperature, the same approach can be used to approximate racemization rates at higher temperatures.

PROCEDURE 2: Dynamic HPLC

Stage 1: Identifying a suitable Analytical HPLC Method ●Timing 1-3 h

1. Weigh approx. 1 mg of the racemic atropisomeric compound (e.g., *rac-***2**) into a 2 mL glass HPLC vial.

2. Add 1.5 mL of Hexane and agitate gently. If the sample remains undissolved, add the minimum volume of 2-propanol required to achieve dissolution. Polar compounds can be dissolved in neat 2-propanol.

3. Carry out standard analytical HPLC runs (isocratic solvent composition; 1 mL/min; 5 μL injection volume) aiming to achieve separation of the two enantiomer peaks with a

plateau region in between. A full description of this step is beyond the scope of this article, but we have found the following general principles helpful when trying to separate the enantiomers of novel chiral compounds.

- Begin by selecting any chiral column and carry out isocratic runs with various ratios of hexane/2-propanol until a retention time of approximately 10 min is obtained.

- Using the same eluent ratio, evaluate all other chiral columns that are available and select that which gives the best separation of enantiomers.

- If some enantiomer separation is evident in some runs, but none show a clear plateau, select the sample with the best separation and run again with a less polar eluent mixture.

- The peak shape will be dramatically affected by temperature. If a single broad peak is present, it is a good idea to chill the column compartment to encourage formation of a batman peak (most HPLC systems enable chilling to ~5 °C). Conversely, if the plateau is very close to the baseline, it may be worth warming the column compartment (note that HPLC columns have different maximum operating temperatures, and it is essential to check the manual before attempting any runs at elevated temperature).

- If adequate separation still cannot be obtained, then try another solvent system (e.g., heptane/*tert*-butyl methyl ether).

After applying the steps above, compound **2** gave an analytical HPLC spectrum with a pronounced Batman effect at 40 °C (Fig. 14).[11]

? TROUBLESHOOTING

Stage 2: Analysing the Raw Data with DCXplorer ● *Timing <20 min*

4. Export a copy of the raw HPLC data as an ASCII file format document (for example a .DAT, .TXT or .CSV document). How this is done will depend on the particular HPLC software that is installed. However, the aim is to generate a two-column text file containing values for time in column one and values for UV absorption in column two.

5. Save a local copy of this text file on the PC using a file name that precisely identifies the data such as "compound 2 HPLC data 40 deg.CSV".

6. Download a copy of DCXplorer using [the following link](https://www.cup.lmu.de/oc/trapp/tools.html) (https://www.cup.lmu.de/oc/trapp/tools.html) .[24]

7. Open DCXplorer and using the tab on the left, navigate to the folder where the file is saved e.g., "Procedure 2". Once the folder is selected, the data file should become visible in the tab at the bottom left (Fig. 15a).

   ? TROUBLESHOOTING

8. Left-click the file name and the full HPLC spectrum should appear in the main window. Left-click and drag over the spectrum to zoom in on the desired peak (Fig. 15b).

   Note: it is possible to return to the full spectrum by double clicking anywhere in the tab showing the HPLC trace.

   ? TROUBLESHOOTING

9. With the peak centred in the display, click the play button to perform the calculation (Fig. 15c)

   Δ CRITICAL STEP At this point it is essential to check the software has identified the peak properly. Four labels in yellow boxes, should have appeared on the spectrum, one at the top of each peak along with further labels at the outer edge of each peak at half height (see Fig. 15d). A horizontal blue line should be present at the centre of the plateau region. If the peak picking does not look like this, zoom out and then zoom back in on the peak attempting to centre it as symmetrically as possible in the display.

   ? TROUBLESHOOTING

10. Find the value for k_{ent} ; it is displayed in the lower tab labelled "**k1[1/s]**". For compound **2**, a value of $k_{ent} = 2.55 \times 10^{-3}$ s^{-1} was obtained (Fig. 15d).

? TROUBLESHOOTING

11. Multiply this value for k_{ent} by two to access k_{rac}:

$$k_{rac} = 2k_{ent} = 2 \times 2.55 \times 10^{-3} = 5.11 \times 10^{-3} s^{-1}$$

12. Substitute this value into eq. ii to calculate the corresponding racemization half-life:

$$t_{rac}^{1/2} = \frac{ln2}{k_{rac}} = \frac{0.693}{5.11 \times 10^{-3}} = 136 \text{ s} = 2.3 \text{ min}$$

13. Substitute the value of k_{ent} along with the temperature of the column oven (for compound **2**, this was 313.15 K) into the Eyring equation to calculate the Gibbs activation energy ($\Delta G^{\ddagger}$). For example, for compound **2**:

$$\Delta G^{\ddagger} = RTln\left(\frac{k_B T}{k_{ent}h}\right) = 8.314 \times 313.15 \times ln\left(\frac{1.381 \times 10^{-23} \times 313.15}{2.55 \times 10^{-3} \times 6.626 \times 10^{-34}}\right)$$

$$= 9.24 \times 10^4 \text{ J/mol}$$

$$= 92.4 \text{ kJ/mol} = 22.1 \text{ kcal/mol}$$

14. If required, use this value to estimate $t^{1/2}{}_{rac}$ at room temperature, as described in Procedure 1.

15. (optional) Repeat the entire Procedure (steps 1-14) several times with different column oven temperatures. This allows calculation of $\Delta G^{\ddagger}$ at different temperatures and construction of an Eyring plot if required.

PROCEDURE 3:

Stage 1: Sample Preparation and Room Temperature 1D NMR ● Timing 30 min

1. Weigh ca. 10-50 μmol of the compound (e.g., **4**) into a 1.75 mL Glass Screw Top Vial.

2. Add 0.5 mL d$_8$-toluene and agitate gently to dissolve.

   ? TROUBLESHOOTING

3. Use a Pasteur Pipette to transfer the solution to an NMR tube and cap the tube.

4. Record an initial 1D NMR spectrum of the relevant nuclei at a convenient temperature.

<CRITICAL STEP> In diarylamine **4** it is the methyl groups, Me$_a$ and Me$_b$, which are in exchange (Fig. 16) so the rate is most easily extracted from the ^{1}H NMR data (^{13}C NMR would also be possible but would be time consuming due to longer experiment times). For other compounds, it might be more appropriate to analyze a different NMR active nucleus.

   ? TROUBLESHOOTING

5. Examine the initial 1D spectrum to determine which peaks are exchanging and estimate which exchange regime the molecule is in. In the case of **4**, the peaks at δ = 1.06 and 1.12 ppm in the ^{1}H spectrum at 20 °C are broader than the others in the system (Fig. 16). It is possible to quantify this observation from the line width at half height (LWHH) which, for these peaks, is several times that of the others, indicating specific broadening due to exchange.

Stage 2: Acquiring VT-NMR Spectra ● Timing 0.5-3 h

6. Choose an appropriate deuterated solvent for the experiment. It is important that the chosen solvent gives good peak dispersion (i.e., separation between multiplets) and that the solvent will not freeze or boil within ±30 °C of the coalescence temperature. The methyl groups of **4** coalesce between 20 and 30 °C (Fig. 17) so a suitable range over which to acquire spectra would, perhaps, be –30 to +50 °C. This range is well within the melting and boiling points of d$_8$-toluene and there is no peak overlap within the area of interest (0.5-1.5 ppm). Temperatures further than 40 °C from coalescence will give

data points with larger uncertainties that may not aid analysis. They can, however, be useful when it comes to understanding the change in chemical shift as a function of temperature (this is independent of exchange) and for visualizing peaks under conditions where no exchange is happening.

? TROUBLESHOOTING

7. Choose the number of temperature increments. For quick analyses, one data point every 10-20 °C is sufficient, however, if accurate data is required (e.g., for an Eyring-Polanyi plot) a data point every 5 °C <u>near coalescence</u> is strongly recommended to minimize the uncertainty in the rate. It is not necessary to obtain a spectrum at the exact coalescence temperature as those nearby will contain enough information.

<CRITICAL STEP> It is important to leave the sample in the spectrometer long enough for the sample temperature to equilibrate for accurate rate estimations. Five to ten minutes should generally prove sufficient.

8. Import the relevant spectra into TopSpin. If the spectra were acquired on a Bruker spectrometer, this is simply a matter of dragging and dropping the files into TopSpin. If they were acquired on another system, they will need to be converted before importing them.

? TROUBLESHOOTING

Stage 3a: Approximating the Exchange Rate by Simple Manual Calculation ● *Timing <5 min*

9. Visually inspect the NMR data collected at different temperatures and note the temperature at which coalescence is taking place (e.g., for compound **4**, Fig 17, $T_c \approx$ 30 °C).

10. Select the window with the lowest temperature spectrum and measure the distance in Hz between the centers of the exchanging peaks (note that in systems where second-order effects are present this may not be the average chemical shift for a multiplet). For diarylamine **4** a separation Δv = 39.1 Hz was measured from the data in Fig 16 at –30 °C.

11. Substitute this value for Δv into eq. vi to calculate the approximate rate of enantiomerization at the coalescence temperature (~30 °C):

$$k_{ent} \text{ at } T_c \approx \frac{\pi \Delta v}{\sqrt{2}} \approx \frac{3.14 \times 39.1}{1.41} \approx 87 \text{ s}^{-1}$$

<CRITICAL STEP> Given the peaks are not singlets (they are non-mutually coupled doublets) equation vi will only give an approximate estimation of the rate.

12. (optional) Substitute this value for k_{ent} into the Eyring equation to estimate the Gibbs activation energy ($\Delta G^{\ddagger}$), and, if required, use this value to approximate the rate of enantiomerization at room temperature.

Stage 3b: Calculating the Exchange Rate by Line Shape Analysis ● *Timing 0.5-5 h*

13. Load one of the spectra in topspin. Type "dnmr" into the bar at the bottom of the screen. Alternatively, under "Analyse" and "Line Shapes", select "Fit Dynamic NMR Models (dnmr)".

14. Select the "SpinSystem" tab from the box that has just appeared. Click "Add" near the bottom (Fig. 18b).

15. Click "Nucleus" and then "Add" once for every peak that is in exchange. For **4**, the only peaks undergoing exchange in the ^{1}H spectrum are the Me_a and Me_b doublets at 1.06 and 1.14 ppm, so click "Add" twice.

16. Enter the exact chemical shifts for the exchanging peaks, 1.0598 and 1.1380 ppm, into the boxes labeled "Nu(iso)" under the "Nuc1" and "Nuc2" tabs, respectively.

17. Enter the coupling constants into the boxes labeled "JX". In this case, both methyl groups are coupled to a methine group that has not yet been defined. As such, it must be created before the coupling constants can be added. Click "Nucleus" and "Add". Set the "Nu(iso)" value to 3.1492 ppm. Coupling constants are set from the higher number nuclei to the lower, i.e., the "Nuc1" to "Nuc3" $^3J_{HH}$ coupling constant is "J1" under the "Nuc3" tab. In this system, the coupling constant between the central methine and each of the methyl groups is identical so "J1" and "J2" can both be set to 6.9 Hz.

    ? TROUBLESHOOTING

18. Check the ratio between the relevant nuclei seen in the spectrum matches the actual ratio of the nuclei in the compound. In this case, there are six methyl protons for every methine proton. It is possible to group nuclei provided they are magnetically equivalent (i.e., a single peak or multiplet), by combining the nuclear spins. In the situation above, the "Pseudospin" value for both methyl groups is 1.5 (n * I = 3 * 0.5) and 0.5 for the methine group. This now reflects the ratio of intensities between the peaks.

19. Line shape is strongly affected by both T_2 and exchange – to get an accurate estimation of the exchange, the natural line broadening as a function of T_2 must be accounted for. The most straightforward approach is to assume that all peaks in the spectrum will have a similar T_2; obtain the underlying line width by measuring a line width for a non-exchanging peak in the spectrum and apply this value to the exchanging peaks. In this example, the line shape of the methine quintet is unaffected by exchange and can be used as the line width reference. The LWHH is approx. 2.3 Hz. Enter this into the box labeled "LB" (line broadening).

    ? TROUBLESHOOTING

20. Change the value in the box labeled "Intensity" until the approximate intensity (height of the peaks) matches that of the real spectrum. Alternatively, click and drag the button the button labeled A in Fig 18a. Dragging up and down will increase or decrease the intensity, respectively.

21. (situational) For systems with unequal populations, add a "molecule" for each non-equivalent conformation under the "Molecule" tab. Enter the molar ratio, or population, of each conformer in the box labelled "X" under the respective tab. Successive populations will be automatically calculated from the "SUMX" value; change this is if the sum of all populations is not 1. Enter -1 in the box labelled "Stch.coef." for the starting material(s) and 1 for the products. Populations can be linked by giving them the same "In X GROUP" value. For the case in question, the starting material (**4**) and product (*ent*-**4**) have identical spectra. As a result, the molar fraction is 1 and the "Molecule" tab can be ignored.

22. Click "Reaction" and then "Add" for each exchange process occurring – in this case, there is a single rate leading to the exchange of Me_a and Me_b so "Reac1" will suffice. Set the value of "Exchanges" to the number of exchanging peaks, i.e., 2 for **4** (Fig. 18c).

23. Leave 1 in the "#1 From" box; it is the first exchanging peak. Enter 2 in the "To" box; "Nuc1" is exchanging magnetic environments with "Nuc2".

24. Enter 2 and 1 in the "#2 From" and "To" boxes, respectively, as these correspond to the reverse process.

25. Change the rate in the box labeled "k" until the line shape looks somewhat like that of the real peaks (it is important to always change the initial value as it is <u>not</u> showing 50 Hz).

26. Choose the range over which the software will optimize. The only peaks of interest in this example are those at ca. 1.06, 1.14, and 3.10 ppm, therefore, click "Spectrum" to the left of "SpinSystem". At the bottom of that tab, click "Edit Range" and enter 1.2

under "From" and 1 under "To". Then click "Add" and enter 3.25 in the new "From" box and 3.05 in the new "To" box. Click "OK" and close the window. There should now be red bars beneath the two exchanging peaks and methine quintet. The value for "Best overlap(%)" on the "SpinSystem" tab should also be significantly greater as the program is considering only the peaks that have been defined and ignoring everything else.

27. Save the system in order to revert to a good starting point later. Click the floppy disk icon on the far right of the DNMR menu bar above "SpinSystem" and enter a descriptive name that will be clear later what it was. Only numbers, letters, underscores, and hyphens may be used in the name – spaces and symbols are not allowed. Click "OK" to save the file. To reopen it, click the folder to the far left of the same menu and then select the relevant file.

28. (optional) Tweak the variables manually until a satisfactory fit is found. This may be based upon visual interpretation or the value for "Best overlap(%)".

29. Alternatively, click the green arrow to automatically search for a better fit. Any variable that does not have a tick mark in the box to its left <u>will not be optimized</u> (Fig. 18b). It is also possible to decide the "Step Length" by clicking the relevant button toward the bottom of the DNMR box. Check the step length for every optimized variable as it may be set to 0 and will therefore not optimize. Click "Return" to save the step length. After several iterations without substantial change to the value for "Best overlap(%)" or after reaching 1000 cycles, the software will stop and display a message indicating it is complete. If the software reached 1000 cycles, repeat this step until it converges before 1000 cycles.

? TROUBLESHOOTING

30. Save the system with an appropriate name to differentiate from the starting point. Record the rate estimated using either the manual or automatic iterative approach in an Excel document (or your preferred data handling program).

<CRITICAL STEP> If an accurate value for the activation energies is required, then it is necessary to find the range of frequencies over which the line shape can be considered accurate to determine the uncertainty of the measurement as there will always be a degree of uncertainty in the estimated rate.

31. Decrease the exchange rates ("Reac1", "Reac2", etc.) until the calculated line shape does not match the experimental spectrum, this is the lower limit of the rate. Do the same but increasing the exchange rates instead to obtain the upper limit. Record these rates as in step 30.

TIMING

PROCEDURE 1: Kinetic Analysis of the Racemization of an Enantioenriched Sample:

Stage 1: Steps 1-3, Optimizing an Analytical HPLC Method: 1-3 h

Stage 2: Steps 1-3, Obtaining an Enantioenriched sample ●Timing 30 min

Stage 3: Steps 1-3, Monitoring the Rate of Racemization ●Timing highly variable – 2 hours to 2 weeks depending on sample.

PROTOCOL 2: Dynamic HPLC

Stage 1: Steps 1-3, Identifying a suitable Analytical HPLC Method ●Timing 1-3 h

Stage 2: Steps 1-3, Analysing the Raw Data with DCXplorer ●Timing <20 min

PROCEDURE 3:

Stage 1: Steps 1-3, Sample Preparation and Room Temperature 1D NMR ● Timing 30 min

Stage 2: Steps 1-3, Acquiring VT-NMR Spectra ● Timing 0.5-3 h

Stage 3a: Steps 1-3, Approximating the Exchange Rate by Simple Manual Calculation ● Timing <5 min

Stage 3b: Steps 1-3, Calculating the Exchange Rate by Line Shape Analysis ● Timing 0.5-5 h

TROUBLESHOOTING:

Troubleshooting advice for Procedures 1-3 can be found in Tables 1-3 respectively.

Table 1│Troublehooting advice for Procedure 1			
Step	Problem	Possible reason	Solution
3	No peaks observed in analytical HPLC spectrum.	Lack of a UV-Vis chromophore.	Carry out analysis on a different product containing a UV-Vis absorbing functional group (e.g., an aromatic ring). Alternatively use a different analytical method such as gas chromatography.
	"Batman-shaped" peak observed by analytical HPLC.	Racemization is relatively rapid on the HPLC timescale.	Carry out analysis described in Procedure 2.

	Single peak observed by analytical HPLC after all columns have been tested.	Racemization is too rapid on the HPLC timescale.	Reanalyse the sample with the column oven set to the lowest possible temperature (e.g., 5 °C) and see if more resolution appears. If not, analyse by VT-NMR (see Procedure 3).
9	Compound is still racemic after semi-preparative HPLC.	Racemization is occurring rapidly.	Perform another semi-preparative run and reanalyse the sample as quickly as possible. If it is still racemic, it is likely racemization is very rapid. Record another analytical HPLC spectrum with the column oven set to a higher temperature (e.g., 40 °C). It is likely that a Batman-shaped peak will be observed. See Procedure 2.

	Analytical HPLC trace has low intensity and cannot be accurately integrated.	The sample produced by semi-preparative HPLC is too dilute.	Carry out several more identical semi preparative runs, combine the product and remove the solvent using a rotary evaporator. Then redissolve to make a more concentrated sample and analyse by HPLC.
17	A very large peak dominates the HPLC spectrum. The analyte signals can no longer be integrated.	The high-boiling solvent used for the racemization study is UV active and coelutes with the desired analyte.	Try a different HPLC eluant system. Alternatively, replace the high-boiling solvent with an alternative one which is less UV active (e.g., decane).

Table 2 | Troublehooting advice for Procedure 2

Step	Problem	Possible reason	Solution
3	No peaks observed in analytical HPLC spectrum.	Lack of a UV-Vis chromophore.	Carry out analysis on a different product containing a UV-Vis absorbing functional group (e.g., an aromatic ring).

	Single peak observed by analytical HPLC after all columns have been tested.	Racemization is too rapid on the HPLC timescale.	Measure a new HPLC injection with the column oven set to the lowest possible temperature (typically 5 °C) and see if peaks become resolved. Note that if the HPLC system does not have an option for column cooling, freestanding column chillers can be purchased that are compatible with any HPLC system. If the sample still remains as a single peak, attempt analysis by VT-NMR (see procedure 3).
7	Data file does not appear in the tab at the bottom left.	Data has been saved with an incorrect file type.	Open the raw datafile and copy the text. Open a blank notepad page from the start menu. Paste the data and then click *Save As…* Save the data as a .TXT file. This file

			should be visible in DCXplorer.
8	No spectrum appears when the file is clicked, or an error message is displayed saying **"Gleitkommaüberlauf"**.	The data in the text file is not in the correct format. For example, there are too many columns of data.	Copy the data into a blank Microsoft Excel spreadsheet (or extract the data directly to Excel from the HPLC software if there is an option to do so). Amend the format so that there are only two columns, with the left column containing the retention times, and the right column containing the corresponding intensities (e.g., the values in mAU recorded by the HPLC). No column headings are required. Highlight the data containing cells and press copy. Then open a blank Notepad page from the start menu. Click paste, and the data should appear in two

columns, with each pair of time and intensity values separated by a space. Save this file as .TXT file. The spectrum should now be viewable in DCXplorer.

| 9 | After experimenting with the zoom extensively the software is still unable to label the spectrum properly. Labels on the spectrum are missing, unsymmetrical and/or clearly in the wrong place. | (a) The data does not display a clear enough Batman profile to be recognised by the software. | This typically occurs with samples that display extreme Batman profiles that are very close to coalescence into a single peak. Measure a new HPLC injection with the column oven set to the lowest possible temperature (typically 5 °C) and see if peaks become more resolved. Alternatively try other HPLC columns and conditions, as different peak separation may lead to a profile that can be analysed. |

	As above	(b) The spectrum contains an impurity that is preventing successful analysis.	Repurify the compound (e.g., by column chromatography or recrystallization) and carry out fresh HPLC analysis.
10	Where a value for k_{ent} should be displayed, the software instead says "**No rate constant calculated!**"	It is likely that the software has not recognised the peak properly (see rows above).	See discussion in rows above.

Table 3 │ Troubleshooting advice for procedure 3			
Step	**Problem**	**Possible reason**	**Solution**
2	No peaks in NMR spectrum.	Concentration is too low.	Use more material when preparing the sample. If more material is unavailable, then increasing the number of scans may help.
4	Every peak has a similar LWHH.	The system is not in the intermediate exchange	Run another spectrum at a different temperature.

		regime and instead in either the fast or slow regimes.	If the number of peaks can be explained by the exchanging peaks having coalesced, then one may be in the fast exchange regime and will need to run a spectrum at a lower temperature. If the opposite is true, then one will need to raise the temperature of the next spectrum. An EXSY spectrum can also be used if in the slow exchange regime as positive cross-peaks will reveal exchanging environments.
6	There are peaks overlapping with the exchanging peaks.	The solvent does not give good peak dispersion.	Try an alternative NMR solvent. Solvent polarity, hydrogen-bonding ability, and aromaticity can have a significant impact on peak dispersion.

6	Line broadening is not observed, even near the freezing/boiling point of the solvent.	The solvent cannot reach the temperatures needed for coalescence.	Try a new solvent with a higher boiling or lower freezing point as appropriate. Suggestions can be seen in the Materials section above. Alternatively try analysis by another method (e.g., Procedure 2).
6	Temperatures above 80 °C are required.	Coalescence does not occur below 80 °C with the chosen solvent.	Use a minimal amount of solvent to reduce temperature gradients.
6	Using d_6-DMSO as a solvent gives very broad line widths.	d_6-DMSO increases the T_2 of many compounds and this causes line widths to broaden more than other solvents.	Try alternative solvents. If nothing gives a better combination of temperature range and polarity, then continue with d_6-DMSO; results will likely still be good enough.

| 8 | The spectra need to be converted before opening in TopSpin. | The spectra were not acquired on a Bruker spectrometer. | Type "open" into the bar at the bottom of the screen or click "Open" from the main dropdown menu. Click "Open NMR data stored in special formats". From the dropdown menu, select the appropriate file type, e.g., for Varian data choose VNMR. Click "Browse" to search through your files for the spectrum, select it and click "OK". At this point TopSpin will suggest a directory within the TopSpin folder itself; if it will be helpful to have all future VT-NMR data in one folder then click "OK". If you would rather separate the VT-NMR analyses for different compounds, use your file explorer window to create a new folder in |

the same directory as the raw data for each compound titled, e.g., "VT-NMR TopSpin Analysis". Selecting the TopSpin window again, paste the file path for this new directory into the "DIR=" bar and click "OK".

| 17 | The line shape for the exchanging peaks is too complex for deconvolution. | There are many coupling constants complicating the peak shape, for example, in an ABX_3 system. | If another pair of diastereotopic signals are available, use those. Alternatively, the peak shape may be simplified using selective decoupling. |
| 19 | All the defined peaks are involved in the exchange process and will not give a line width unaffected by exchange. | | Another peak must be used to approximate the natural line broadening. Look for an alternative peak that is clearly resolved (it can even be a single peak in a multiplet). The methyl |

singlets at 2.1 ppm in **4** (Fig. 16) are also perfect for this. Create a new spin system and add enough nuclei to represent the peak from which you plan to extract the line width. Make sure both System1 and System2 have the same "LBGROUP" and that they are nonzero. Bear in mind, this is at best an approximation.

| 29 | The system does not converge before 1000 cycles even after multiple restarts. | The system may have found a local minimum that may or may not fit the real spectrum. | If the simulated lineshape appears to accurately represent the real spectrum it may be sufficient to use the rate acquired at this point. If not, change the parameters enough to move out of the local minimum and restart the optimization. |

ANTICIPATED RESULTS

Atropisomeric molecules are set to play an increasingly important role in modern society, with emerging applications in catalysis, materials science and healthcare. By definition, it is possible for an enantiopure atropisomer to racemize by bond rotation and it is essential to evaluate how rapidly this process takes place. This article sets out the three most widely used procedures to measure racemization rates of atropisomeric molecules, namely: (1) kinetic analysis of the racemization of an enantioenriched sample (e.g. Fig. 10-12); (2) dynamic HPLC (e.g. Fig. 14-15); and (3) variable temperature NMR (e.g. Fig. 16-18). Each procedure is illustrated with worked examples, illustrating the requisite experimental techniques, analysis methods and calculations. This article serves as a guide to help researchers new to this area make sense of which procedures to use, the basic theory behind them and exactly how they can be performed.

Data availability: We have previously reported synthetic procedures and data for compounds **1** (ref. 11), **2** (ref. 11) and **4** (ref. 18), which are used to illustrate procedures 1-3.

Author Contributions: The manuscript was jointly devised and written through the contributions of all authors.

Competing interests: The authors declare no competing interests.

Key references using these procedures:

(i)   Costil, R., Sterling, A. J., Duarte, F. & Clayden, J. *Angew. Chem. Int. Ed.* **59**, 18670–18678 (2020). https://onlinelibrary.wiley.com/doi/10.1002/anie.202007595

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FIGURES

Fig. 1│An overview of atropisomerism and configurational stability. a, Selected examples of various important atropisomeric scaffolds. **b,** A representative example depicting the enantiomerization of a generic axially chiral biaryl along with a summary of the synthetic strategies available to prepare atropisomeric molecules. **c,** The difference between enantiomerization (a reversible microscopic process) and racemization (an irreversible macroscopic process).

Box 1│Key equations i-iii, defining kinetic parameters for configurational stability of atropisomers: R is the gas constant (8.314 J K^{-1} mol^{-1}); T is the temperature in Kelvin; k_B is the Boltzmann constant (1.381×10^{-23} J K^{-1}) and h is Planck's constant (6.626×10^{-34} J s).

Fig. 2│Different timescales for bond rotation and definitions of atropisomerism. Illustration of the relationship between $\Delta G^{\ddagger}$ and $t^{1/2}_{rac}$ at 25 °C. The LaPlante classification system defines three categories of atropisomer (classes 1, 2 and 3)

depending upon configurational stability. Oki's definition states that any compound with $t^{1/2}_{rac} > 1000$ s is atropisomeric.

Fig. 3│ Kinetic Analysis of the Racemization of an Enantioenriched Sample. a, An overview of the method: preparative HPLC resolution of a racemic atropisomer (stage 1), followed by a series of analytical measurements of enantiomeric ratio over time (stage 2). **b**, A plot of enantiomeric excess over time shows exponential decay according to equation iv. **c**, It is generally more convenient to plot ln(1/e.e.) vs time to obtain a straight line with gradient = k_{rac}. e.e. = enantiomeric excess at a given time (t), $e.e._{t=0}$ = the initial enantiomeric excess.

Fig. 4│ Representative example. Axially chiral enol ether **1** has been chosen to illustrate the experimental procedure for determination of racemization rate and associated barriers by HPLC analysis.

Fig. 5│Dynamic HPLC and calculations using the unified equation. a, Schematic examples of HPLC chromatograms obtained in three scenarios: (i) enantiomerization is rapid on the HPLC timescale; (ii) enantiomerization occurs at a comparable rate to the HPLC timescale leading to a "Batman-shaped" peak; (iii) enantiomerization is slow on the HPLC timescale. **b**, The line shape can be used to calculate k_{ent} according to the unified equation, where t_R^A and t_R^B = the retention times of peaks A and B respectively; h_A, h_B and h_P = the heights of peaks A, B and the plateau region respectively; w_A and w_B refer to the widths at half-height of peaks A and B respectively; N = the number of theoretical plates; $\sigma_A = w_A/\sqrt{8ln2}$; $\sigma_B = w_B/\sqrt{8ln2}$. Fortunately, Trapp and co-workers

have designed freely available software that can perform such calculations automatically from raw HPLC data.

Fig. 6│ Representative example. Diarylamine **2** has been chosen to illustrate the experimental procedure, which displays unusual C–N–C atropisomerism on the HPLC timescale.

Fig. 7│ Schematic example of an axially chiral biaryl. a, Enantiomerization exchanges the environments of the diastereotopic protons of Me_a and Me_b. **b,** Depending upon how the rate of the enantiomerization process (k_{ent}) compares to the NMR timescale, different NMR spectra will be observed.

Fig. 8│ Representative example. Diarylamine **4** has been chosen to illustrate the experimental procedure.

Fig. 9│Workplan for analysing the barrier of a completely unknown compound. Begin by carrying out HPLC on a chiral stationary phase and observing the peak shape. Then follow the further steps shown to determine which procedure should be carried out.

Fig. 10│Analytical HPLC data for illustrative compound 1. Analytical HPLC data obtained after method optimization, showing baseline separation between enantiomer peaks suitable to progress to the next stage.

Fig. 11│Semi-preparative HPLC isolation of a single enantiomer. a, Schematic example showing HPLC data for a generic racemic atropisomer, with pink band indicating the time interval targeted for collection. **b**, If the HPLC system is not equipped with a fraction collector, manual collection should be performed with a short length of HPLC tubing attached to the detector outlet, collecting in a clean 2 mL HPLC vial.

Fig. 12│Monitoring atropisomer racemization by HPLC at elevated temperature. a,

Experimental setup for racemization of a *m*-xylene solution of **1** at 139 °C. **b,** Analytical

HPLC data showing successive reinjection of a single enantiomer sample of **1** obtained

by semi-preparative HPLC over the course of 4 hours (each injection with Chiralpak ADH,

99:1 hexane/2-propanol, λ = 281 nm, 5 μL injection). **c,** Integration of this data produced

the following results. For compound **1** a plot of time (in seconds) vs ln(1/e.e.) produces a

straight line with a gradient of $1.82 \times 10^{-4} \, \text{s}^{-1} = k_{rac}$.

Fig. 13│Using ΔG‡ to estimate racemization rates at other temperatures using the

"Eyring equation calculator". The value for $\Delta G^{\ddagger}$ can be input in either kJ/mol or kcal/mol

along with a temperature in Kelvin. The half-life displayed is for enantiomerization and

must be divided by two to access $t^{1/2}_{rac}$. The figure shows calculated values for compound

1 at: **a,** 412.15 K (the temperature of the racemization study) and **b,** 298.15 K (room

temperature).

Fig. 14│Analytical HPLC data for illustrative compound *rac*-2. Analytical HPLC data

obtained after method optimization for diarylamine **2** displaying a pronounced Batman

effect.

Fig. 15│Analytical HPLC data for illustrative compound *rac*-2. Step-by-step instructions

on using DCXplorer to determine enantiomerization rates. **a,** select the desired data set.

b, left-click to zoom into the region of interest. **c,** select play to run a simulation. **d,** read

the resulting value of k_{ent}.

Fig. 16│^{1}H NMR spectrum of 4 at 20 °C in d$_8$-toluene. The peaks at 1.06 and 1.12 ppm

correspond to the diastereotopic isopropyl methyl groups and the signal at 3.14 ppm

corresponds to the isopropyl methine. The peaks at 2.00 and 2.04 ppm relate to the tolyl

groups and the multiplet at 2.09 is the residual solvent peak.

Fig. 17│^{1}H VT-NMR spectra of 4 in d$_8$-toluene. The spectra above were acquired

in 10-degree increments between –30 °C and +50 °C. By +30 °C the peaks of Me$_a$

and Me$_b$ have just coalesced, while by +50 °C the line shape has begun to resolve

into a doublet, indicating they have become equivalent on the NMR timescale.

Fig. 18│DNMR in TopSpin. a, Various tasks and NMR parameters can be changed

directly from the DNMR menu bar. The parameters that can be changed by

clicking and dragging with a mouse are in green and labeled A-F. **b,** A-F can also

be manually fine tuned in the main DNMR window under the "SpinSystem" tab.

Other parameters can also be set in this window. **c,** The rate of exchange and

specific exchange parameters can be found under the "Reaction" tab.

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