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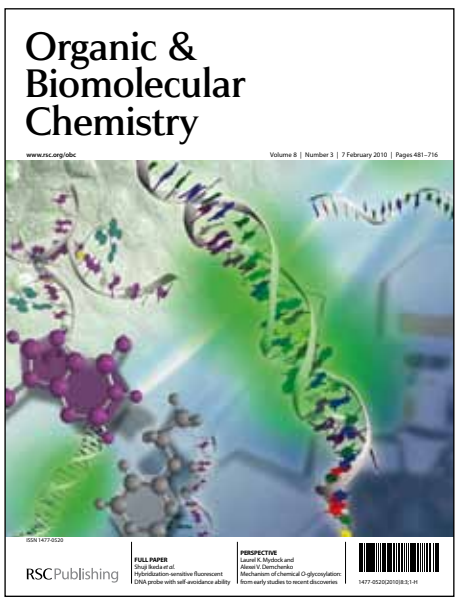

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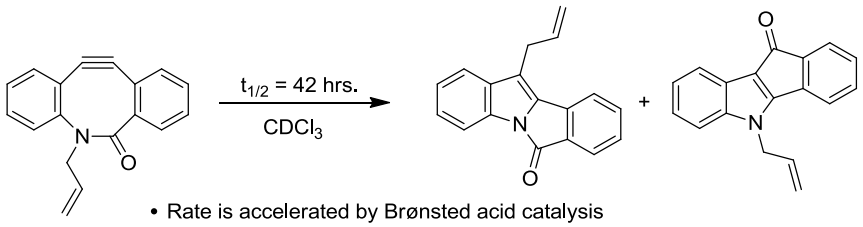

- Rate is accelerated by Brønsted acid catalysis
- Rearrangement observed for a derivative commonly used for bioconjugation
- 3 X-ray structures

Rearrangements and addition reactions of biarylazacyclooctynones and the implications to copper-free click chemistry

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Highly strained biarylazacyclooctynone (BARAC) and analogous bioconjugation reagents were shown to undergo novel rearrangement and addition reactions leading to tetracyclic products. This may limit their practical applicability as bioorthogonal reporters for imaging biomolecules within living systems.

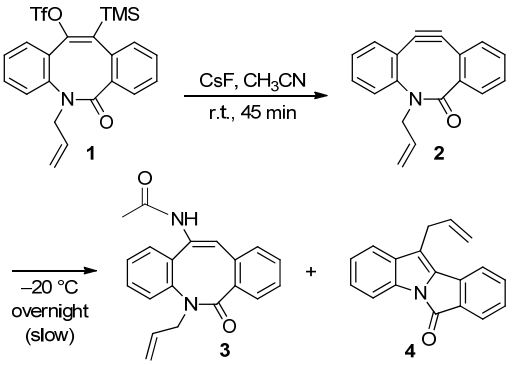
Introduction

Bioorthogonal reactions allow tracking of nucleic acids, lipids, and glycans, and post-translational modifications that are not accessible using conventional genetically encoded reporters.[1-4] Azide-based bioorthogonal reactions such as Staudinger ligations with triaryl phosphines,[5, 6] Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC),[7, 8] and strain-promoted azide-alkyne cycloaddition (SPAAC)[9] have been most widely employed. SPAAC has been shown to be biocompatible,[10] and has undergone several mechanistic modifications to improve its applicability for bioconjugation. Rate enhancements have typically been achieved with modifications to the alkyne component, through incorporation of propargylic *gem*-difluoro groups (difluorocyclooctyne, DIFO, k = 0.076 $M^{-1}s^{-1}$),[11, 12] benzannulation (dibenzocyclooctyne, DIBO, k = 0.057 $M^{-1}s^{-1}$),[13] cyclopropyl fusion (bicyclononyne, BCN, k = 0.140 $M^{-1}s^{-1}$),[14] increase in the number of sp^2 hydridised carbon atoms in the ring (keto-DIBO, k = 0.26 $M^{-1}s^{-1}$),[15] or by reducing the strained alkyne ring size through heteroatom substitution (tetramethylthiacycloheptyne, TMTH, k = 4.0 $M^{-1}s^{-1}$).[16] Another important modification to SPAAC has been to increase the hydrophilicity and pharmacokinetics of the cyclooctyne component for *in vivo* applications within living organisms. Alkynes such as dimethoxyazacyclooctyne (DIMAC, k = 0.0030 $M^{-1}s^{-1}$),[17] azadibenzocyclooctynes (DIBAC or ADIBO, k = 0.31 $M^{-1}s^{-1}$),[18, 19] and biarylazacyclooctynone (BARAC, 0.96 $M^{-1}s^{-1}$).[20, 21] contain additional nitrogen atoms embedded within the cyclooctyne ring to increase solubility. Alternative 1,3-dipoles, such as nitrones,[22-24] diazoalkanes,[25] and nitrile oxides have also been employed to improve the kinetics of strain-promoted cycloadditions with cyclooctynes.[26, 27]

One of the desired properties of bioorthogonal click reactions is that they occur with sufficiently fast rates. However, in developing reactions with higher rates and better reactivity the possibility of reduced selectivity and competing side reactions becomes problematic. Herein, we report the rearrangement of a BARAC analogue and the kinetic studies thereof. We also determine that the rearrangement is acid catalysed and propose a mechanism for this process. Understanding the rearrangement of azacyclooctynes now makes it possible to predict when BARAC and related analogues can be used successfully for bioconjugation *in vivo*, and why certain analogues may fail.

Results and discussion

We originally sought to synthesize a simplified BARAC analogue containing an allyl linkage for kinetic studies of its reaction with cyclic nitrones, as we anticipated that the absence of the additional stereocenter in the linker moiety of BARAC would simplify characterisation of the resultant cycloadducts. However, treatment of known intermediate **1**[20] with CsF in MeCN for 1.5 hrs, and purification following overnight storage at –20 ˚C did not yield 11,12-didehydro-5-allyl-dibenz[*b*,*f*]azocin-6(*5H*)-one (**2**). Rather, a mixture of (*E*)-*N*-(5-allyl-6-oxo-5,6-dihydrodibenzo[*b*,*f*]azocin-12-yl)acetamide (**3**) and 11-allyl-6*H*-isoindolo[*2*,*1-a*]indol-6-one (**4**) was obtained in a 9:1 ratio, respectively (Scheme 1).

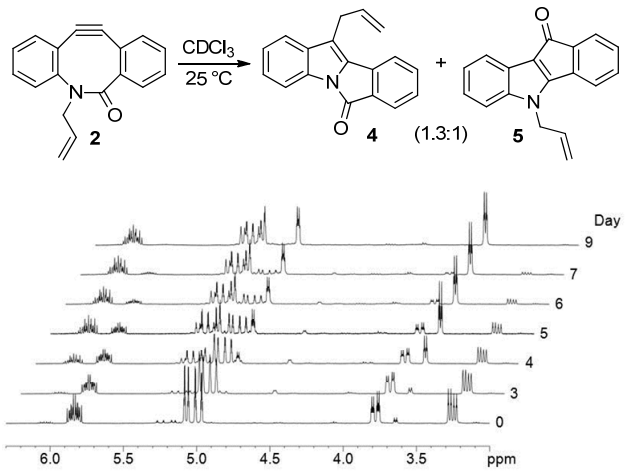


Scheme 1 Synthesis of **2** and first observation of its decomposition into products **3** and **4**.

The *N*-alkyl amide by-product **3** may have been formed by a Ritter reaction,[28] via protonation of the alkyne, electrophilic addition with MeCN, and hydrolysis of the nitrilium ion by H_2O present. It is also noteworthy that an indole product analogous to **4**, has been observed by others during the synthesis of DIBAC.[18] It was presumed that indole formation resulted from 5-*endo-dig* cyclisation to relieve ring strain during CBz-group removal.[18] This observation further supports our findings with BARACs. To determine if the rearrangement of **1** to **4** was reversible, **4** was heated to 78 °C for 1 h, formation of **2** was not observed. Next, CsF (1 equiv.) was added to solution of **4**, to determine if CsF had a role in the reverse reaction, no reaction was observed. Lastly, upon heating **4** in the presence of CsF and TMS-Cl in solution ($CDCl_3$), no reaction was observed. To confirm that formation **4** resulted from rearrangement of the alkyne

intermediate **2**, **1** was reacted with CsF in the presence of an acyclic aldonitrone, the corresponding cycloadduct was isolated 46 % yield (see ESI). These data suggest that **4** was formed irreversibly via rearrangement of **2**.

Next we measured kinetics for the rearrangement of **2** by ^{1}H NMR in $CDCl_3$ (rather than CD_3CN) to avoid competing nucleophilic addition of CD_3CN to the strained alkyne. Scheme 2 shows stacked ^{1}H NMR plots, which illustrate the progression of rearrangement of **2** in neutralised $CDCl_3$. Interestingly, rearrangement of **2** under these conditions provided a mixture of products **4** and **5** (5-allylindeno[1,2-*b*]indol-10(5*H*)-one), formed in a ratio of 1.3:1. No additional by-products were observed by ^{1}H NMR. Plotting the natural logarithm of the peak intensity of the disappearance of **2** as a function of time provides the unimolecular rate constant for the rearrangement ($k_{rear.}$ = 4.54 x 10^{-6} s^{-1}), corresponding to a half-life of ~42 hrs. This has significance to the practical utility of **2** for bioorthogonal reactions, especially in biological studies where azacyclooctynones must be incubated in living systems over prolonged periods of time.[29, 30] The structures of **3**, **4** and **5** were elucidated by X-ray crystallography (Figure 1) and NMR.



Scheme 2 Overlay of ^{1}H NMR spectra showing time-dependant rearrangement of **2** over time in $CDCl_3$ at 25 ± 0.1 °C.

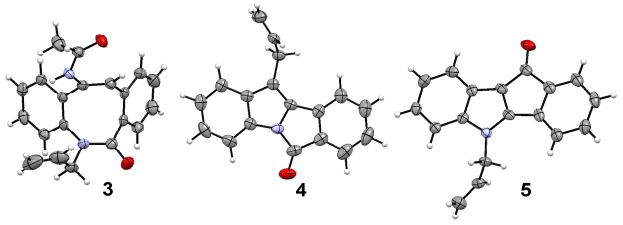
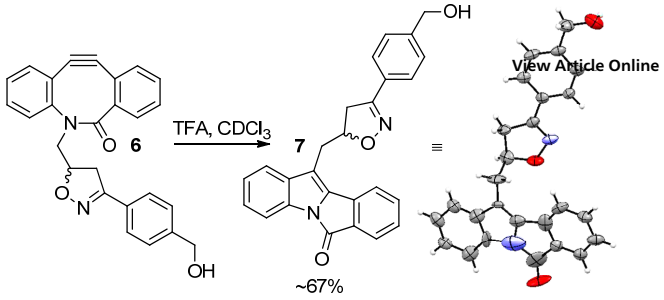


Figure 1 ORTEP diagrams of **3**, **4** and **5** with thermal ellipsoids shown.

We also observe that rearrangement of biarylazacyclooctynones is not exclusive to analogues bearing the allyl linker. It was noted that BARAC containing a 4,5-didehydro-oxazole side chain (**6**) rearranged to the analogous product (**7**). We estimated the half-life of compound **6** to be approximately 5 days in $CDCl_3$. The structure of **7** was elucidated by X-ray crystallography. The ORTEP diagram is displayed in Scheme 3 and in the ESI.



Scheme 3 Rearrangement of **6** to **7**. The ORTEP diagram of **7** is shown.

Having observed that BARAC derivatives **2** and **6** are prone to rearrangement, we evaluated if the rearrangement of **2** can be accelerated in the presence of acid. We hypothesised that protonation at the amide oxygen or the alkyne would facilitate rearrangement. Generally, proton transfers to heteroatoms are fast (diffusion controlled),[31] whereas transfers to carbon are slower, with only a few exceptions.[32] It was anticipated that amide protonation at oxygen would accelerate the hydration of the alkyne, whereas protonation of the alkyne would be expected to accelerate the 5-*endo-dig* transannular cyclisation (*vide infra*). Rearrangement of **2** in $CDCl_3$ in the presence of varying concentrations of TFA was monitored by ^{1}H NMR at 25 ± 0.1 ºC. In the presence of TFA (0-0.3 M), the rate of rearrangement was accelerated as the concentration of acid increased, suggesting the rearrangement is acid catalysed. The slope of the plot of k_{obs} versus [TFA] corresponded to a k_{H+} value of 8.18 x 10^{-3} $M^{-1}s^{-1}$ (Figure 2). We also observe that **6** undergoes acid catalysed rearrangement with a rate constant comparable to that of **2**.

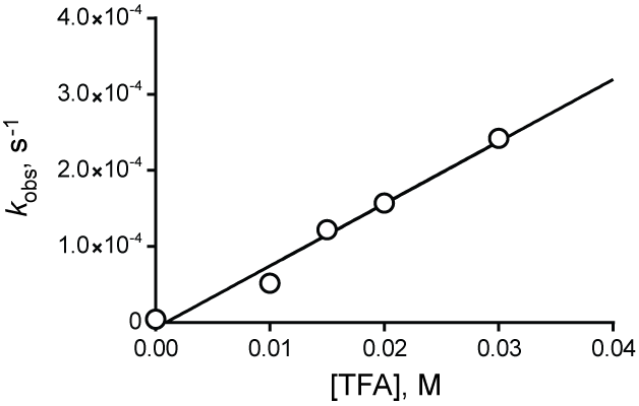
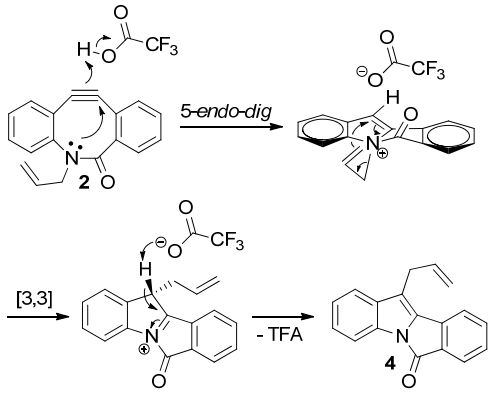


Figure 2 Rate constants for the kinetic studies of rearrangement of BARAC correlated to the various concentrations of TFA in $CDCl_3$ at 25 ± 0.1 ºC.

The highly strained alkyne moiety in **2** is prone to various reactions with nucleophiles present. We speculate that **4** was formed by an intramolecular cyclisation/aza-Claisen rearrangement sequence involving transannular 5-*endo-dig* cyclisation of the endocyclic amide nitrogen (Scheme 4).



Scheme 4 Plausible mechanism for the formation of **4** under TFA catalysis.

This mechanism is supported by the computational study the MM2 energy-minimised geometry of **2** using ChemBio3D Ultra, which shows orbital overlap between the nitrogen lone pair and the alkyne π* molecular orbital (Figure 3).

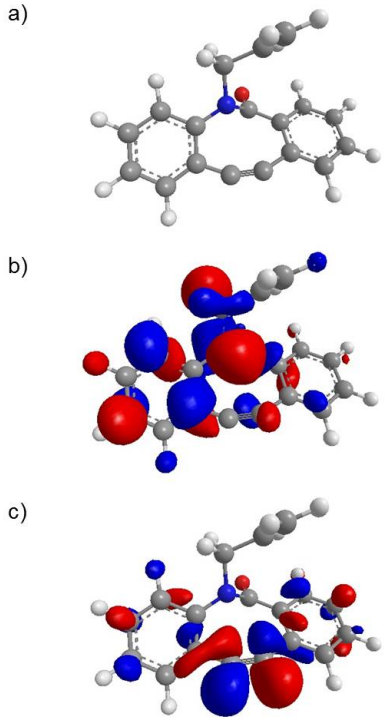
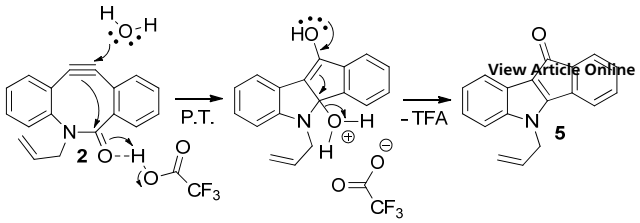


Figure 3 a) MM2 energy-minimised force field results for **2** determined using ChemBio3D Ultra. b) HOMO extended Hückel molecular model calculation results. c) LUMO extended Hückel molecular model calculation results.

This proposed mechanism is also corroborated by a previous account of a Brønsted acid-catalysed 5-*endo-dig* cyclisation of propargylglycine derivatives to afford the corresponding pyrroles.[33, 34] Furthermore, the room-temperature aza-Claisen rearrangement of a quaternary *N*-allyl enammonium salt has been disclosed.[35, 36] The formation of **5** presumably resulted from reaction of the alkyne moiety with residual H_2O present (Scheme 5).



Scheme 5 Plausible mechanism for the formation of **5** under TFA catalysis.

In the literature, cyclooctynes have been reported to undergo nucleophilic addition from cellular nucleophiles such as glutathione[37, 38] or spontaneous homotrimerisation.[39] It is important to consider that by increasing reactivity of BARAC designed for strain-promoted 1,3-dipolar cycloadditions, there is a risk that selectivity and bioorthogonality may be compromised.

Conclusions

In summary, we have shown that BARAC derivatives are prone to novel rearrangements and various addition reactions that could have implications for their practical utility as bioorthogonal reporter groups. We have shown that rearrangement of **2** is accelerated in the presence of acid, and have found that BARAC containing a 4,5-didehydro-oxazole side chain linker (**6**) underwent analogous rearrangement. The difference in rates of rearrangement of these BARAC analogues has enabled us to speculate that the linker group has profound effects on rates of rearrangement. BARAC analogues containing linkers that can form good leaving groups, likely rearrange more readily than those bearing poorer leaving groups. The rearrangements and addition reactions of biarylazacyclooctynones have particular relevance to biological labelling via copper-free click chemistry. At low concentrations of labelling reagents rearrangement could become a competing process and may lower the efficiency of the tagging. It is necessary to slow the rearrangement while keeping the cycloaddition fast. This problem of BARAC rearrangement can be minimised through linkers consisting of poor leaving groups (i.e. primary alkyl groups, exocyclic amide, etc.).[18, 20, 21] Also, the use of BARAC should be avoided in conditions where acid catalysis can occur. The most reactive cyclooctynes will inevitably be further tuned for animal studies where the concentrations of reagents will be low and possibility of side reactions higher. These studies emphasize the delicate balance between reactivity and stability that must be achieved for optimal applications of bioorthogonal reactions and suggests that biarylazacyclooctynones may be reaching the limits of practical applicability as bioorthogonal reporter tags.

Experimental

All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope laboratories. Thin layer chromatography was performed on Analtech Uniplate® silica gel plates (60 Å F_{254}, layer thickness 250 μm). Flash chromatography was performed using silica gel (60 Å, particle size 40–63 μm). LC-MS/MS spectra were obtained using ESI^+. All ^1H and ^{13}C NMR spectra and kinetic

studies were obtained on a Bruker-DRX-400 spectrometer using a frequency of 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C and processed using Bruker TOPSPIN 2.1 software. Chemical shifts are reported in parts per million (δ) using residual $CHCl_3$ resonance as an internal reference (7.26 and 77.0 ppm for ^{1}H and ^{13}C NMR, respectively). The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad signal and J = coupling constants in Hz. The nitrone (**8**),[40] intermediate (**1**) and BARAC (**6**) [41] were prepared according to previously to literature procedure.

Synthesis of 11,12-didehydro-5-allyl-dibenz[*b,f*]azocin-6(5*H*)-one 2

To a mixture of **1** (150 mg, 0.31 mmol, 1 equiv.) and CsF (284 mg, 1.9 mmol, 6 equiv.) was added CH_3CN (1.5 mL) all at once. The solution was stirred vigorously for 1.5 h, and the solvent was removed. The resultant crude oil was immediately purified by silica gel column chromatography (Hx:EtOAc/9:1, R_f = 0.28). The SiO_2 was neutralised with 96:4/Hx:Et_3N prior to purification of **2**. The title compound was obtained as a light yellow oil (59.3 mg, 0.23 mmol, 74 %) and was stored under argon at -80 ºC. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.59 (1 H, dd, J = 6.8, 1.9 Hz), 7.54 (1 H, dd, J = 6.4, 2.8 Hz), 7.4-7.5 (4 H, m), 7.34-7.39 (2 H, m), 5.83 (1 H, dddd, J = 17.1, 10.2, 7.2, 5.0 Hz), 5.07 (1 H, ddd, J = 10.2, 2.5, 1.25 Hz), 4.99 (1 H, ddd, J = 17.0, 2.9, 1.4 Hz), 3.78 (1 H, tdd, J = 15.2, 4.9, 1.6 Hz), 3.26 (1 H, tdd, J = 15.4, 7.2, 1.0 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 176.7, 155.1, 149.2, 133.9, 130.6, 129.5, 129.3, 128.7, 128.1, 127.5, 126.4, 126.0, 122.6, 122.4, 118.2, 109.8, 109.4, 54.7. ESI-MS: Calculated for $C_{18}H_{14}NO$ (M^+) = 260.10, Found 260.1

Procedures for kinetic studies of rearrangement of 2

Rearrangement of 2 under neutral conditions

Precautions were taken as to not introduce acid into the starting material **2**. Non-deuterated chloroform for azeotroping and deuterated chloroform used as solvent in the reaction were carefully neutralised by filtering through a short plug of solid sodium bicarbonate. The NMR tube was sealed and kept in a water bath at 25 ºC. Caution was also taken as to not expose the reaction to light. A ^{1}H-spectra was taken daily until **2** had disappeared completely. The solvent was evaporated *in vacuo* and crude was purified by silica gel column chromatography (Hx:EtOAc/8:2, R_f (4) = 0.6, R_f (5) = 0.3) to yield 4 as light yellow solid (10.7 mg, 0.041 mmol, 53 %) and 5 (8.2 mg, 0.0317 mmol, 41 %) as red solid. **11-allyl-6*H*-isoindolo[2,1-*a*]indol-6-one** (**4**): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.89 (1 H, d, J = 8.0 Hz), 7.77 (1 H, d, J = 7.6 Hz), 7.51 (2 H, m), 7.42 (1 H, d, J = 7.8 Hz), 7.30 (2 H, m), 7.15 (1 H, m), 6.03 (1 H, tdd, J = 16.2, 10.1, 6.1 Hz), 5.20 (2 H, ddd, J = 13.6, 11.5, 1.6 Hz), 3.63 (2 H, td, J = 6.1, 1.6 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 162.4, 135.1, 135.0, 134.8, 134.5, 134.0, 133.7, 133.6, 133.6, 128.3, 26.6, 125.4, 123.7, 121.6, 120.6, 117.3, 116.7, 113.4, 28.9. ESI-MS: Calculated for $C_{18}H_{14}NO$ (M^+) = 260.10, Found 260.2; **5-allylindeno[1,2-*b*]indol-10(5*H*)-one** (**5**): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.81 (1 H, ddd, J = 8.0, 1.3, 0.7 Hz), 7.46 (1 H, ddd, J = 7.0, 1.4, 0.6 Hz), 7.15-7.25 (4 H, m), 7.11 (1 H, ddd, J = 7.1, 1.1, 0.6 Hz), 6.06 (1 H, tdd, J = 17.1, 10.2, 4.8 Hz), 5.29 (1 H, dtd, J = 10.5, 1.8, 0.5 Hz), 5.16 (1 H, dtd, J = 17.1, 1.8, 0.4 Hz), 4.92 (2 H, td, J = 4.7, 1.8 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 185.1, 158.6, 142.4, 141.2, 134.7, 132.0, 131.5, 129.7, 123.6, 123.3, 123.2, 123.0, 120.9, 118.6, 118.0, 115.4, 110.8, 47.4. ESI-MS: Calculated for $C_{18}H_{14}NO$ (M^+) = 260.10, Found 260.1.

Rearrangement of 2 under acidic conditions

Precautions were taken to not introduce acid into the starting material **2**. Non-deuterated chloroform for azeotroping and deuterated chloroform used as solvent in all reactions were carefully neutralised by filtering through a short plug of solid sodium bicarbonate. Fresh batch of neutralised solvent was used for each reaction. A ^{1}H-spectra was taken of each sample without acid for assuring purity of starting material. A calculated amount of stock solution of TFA required to achieve the desired final concentration was added to the solution of **2** in NMR-tube. A ^{1}H-spectra was taken periodically over several hours until complete disappearance of the starting material. All reaction were performed at 25 ± 0.1 ºC using the NMR spectrometer built-in temperature control system.

Kinetic data analysis

The data analysis was identical in all kinetic experiments. The intensity of a characteristic peak of **2** was calibrated to 100 %. The same peak in all spectra in the kinetic trial was integrated using the initial calibration. Natural logarithms of intensity values were plotted against time (in seconds). Only the linear portion of the regression was used to fit a linear trend line, the negative slope of which represented the rate constant for the given experiment at the given concentration of TFA. The rate constants were then plotted against the corresponding TFA concentrations. The slope of the linear trend line fitted to the data points represents the k_{cat} for the rearrangement reaction. For detailed kinetic results see ESI.

Notes and references

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