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Highly Precise Measurement of Kinetic Isotope Effects Using ^{1}H-Detected 2D [^{13}C,^{1}H]-HSQC NMR Spectroscopy

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Supporting Information

ABSTRACT: A new method is presented for measuring kinetic isotope effects (KIEs) by ^{1}H-detected 2D [^{13}C,^{1}H]-heteronuclear single quantum coherence (HSQC) NMR spectroscopy. The high accuracy of this approach was exemplified for the reaction catalyzed by glucose-6-phosphate dehydrogenase by comparing the 1-^{13}C KIE with the published value obtained using isotope ratio mass spectrometry. High precision was demonstrated for the reaction catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *Mycobacterium tuberculosis*. 2-, 3-, and 4-^{13}C KIEs were found to be 1.0031(4), 1.0303(12), and 1.0148(2), respectively. These KIEs provide evidence for a cleanly rate-limiting retroaldol step during isomerization. The high intrinsic sensitivity and signal dispersion of 2D [^{13}C,^{1}H]-HSQC offer new avenues to study challenging systems where low substrate concentration and/or signal overlap impedes 1D ^{13}C NMR data acquisition. Moreover, this approach can take advantage of highest-field spectrometers, which are commonly equipped for ^{1}H detection with cryogenic probes.

Kinetic isotope effects (KIEs) are a powerful tool for the characterization of reaction mechanisms, revealing geometric and electrostatic properties of the substrate at the transition state (TS). The determination of multiple KIEs facilitates computational modeling of the TS, which in the case of enzyme-catalyzed reactions, can serve as a template for the design of TS analogues that may act as potent inhibitors.[1,2]

To be useful in TS analysis, accuracy and precision are critical. Among approaches for the measurement of KIEs, competitive assays utilizing mixtures of isotopologues are superior in both qualities, as the light and heavy isotopologues experience identical reaction conditions within the same sample.[3] KIEs can be calculated from measurements of the fraction of conversion of light isotopologue (F_1) and of the ratio of heavy to light isotopologue before reaction (R_0) and after partial conversion (R), and a fit of eq 1 to the experimental data:

$$R/R_0 = (1 - F_1)^{(1/\mathrm{KIE})-1} \quad (1)$$

Bennet and co-workers[4] established a ^{13}C NMR-based approach that incorporates a ^{13}C "reporter" spin adjacent to the atom whose KIE is to be measured. Whereas the light isotopologue gives rise to a singlet line, the heavy isotopologue yields a doublet for scalarly coupled spin-$^{1}/_{2}$ nuclei (e.g., ^{13}C) or an upfield singlet for nuclei devoid of a spin (e.g., ^{18}O).[a] The signal-to-noise ratios (S/N) and spectral simplicity registered for the ^{13}C-enriched position permit continuous monitoring of isotopic ratios without the need for separation of reactant and product. A drawback, however, is the low intrinsic sensitivity of ^{13}C when compared to ^{1}H NMR,[5] which may necessitate the use of a ^{13}C-enhanced cryogenic probe.[4]

To overcome this limitation, we report here the use of ^{1}H-detected, sensitivity-enhanced[6] two-dimensional (2D) [^{13}C,^{1}H]-heteronuclear single quantum coherence (HSQC) spectroscopy. When considering only the ratio of the ^{1}H and ^{13}C gyromagnetic ratios,[5,7] the combined ^{1}H excitation and detection can potentially increase sensitivity up to 32-fold[b] when compared to 1D ^{13}C NMR.[8] Most importantly, such 2D spectra can be acquired on highest-field NMR spectrometers, which are nowadays commonly equipped with highly sensitive cryogenic probes designed for ^{1}H detection.

It is evident that 2D [^{13}C,^{1}H]-HSQC requires a ^{1}H attached to the ^{13}C reporter (Figure 1); however, since only one of the carbons neighboring the carbon of interest must be bound to a proton, most organic compounds and biomolecules meet this condition. Moreover, KIEs can be measured by following either reactant or product, which further relaxes this constraint.

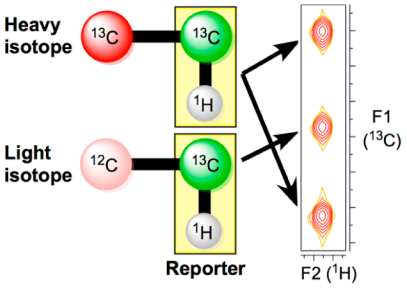

Figure 1. Measurement of ^{13}C kinetic isotope effects by 2D [^{13}C,^{1}H]-HSQC. The ^{13}C−^{1}H reporter gives rise to a cross-peak being a singlet along f1(^{13}C) when adjacent to ^{12}C (pink) but to a doublet when adjacent to ^{13}C (red).

The 2D [^{13}C,^{1}H]-HSQC-based approach was evaluated by applying it to the reaction catalyzed by 1-deoxy-D-xylulose-5-phosphate (DXP) reductoisomerase from *Mycobacterium tuberculosis* (*Mt*DXR) and comparing it with the approach based on

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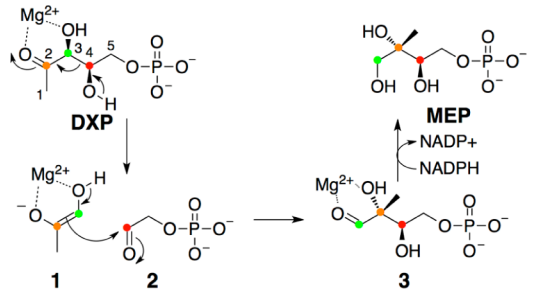
1D ^{13}C NMR. *Mt*DXR is an essential enzyme that controls the first committed step in the non-mevalonate pathway for isoprenoid synthesis in *M. tuberculosis* and other microbes.[9] Since it is absent in mammals, it is a promising target for development of antimicrobial drugs. *Mt*DXR catalyzes the sequential isomerization of DXP to an aldehyde intermediate (**3**) and reduction of **3** by NADPH to yield 2-*C*-methyl-D-erythritol 4-phosphate (MEP), purportedly by a retroaldol–aldol mechanism (Scheme 1).[9,10] The method introduced here was used to evaluate this mechanism for *Mt*DXR and additionally to assess the relative contribution of the two steps involved in isomerization.

Scheme 1. Retroaldol–Aldol Mechanism of DXR-Catalyzed Conversion of DXP to MEP



Two-dimensional [^{13}C,^{1}H]-HSQC was employed for measuring ^{13}C KIEs at carbons 2, 3, and 4. Accordingly, the required [1-^{13}C]-, [1,2-$^{13}C_2$]-, [3-^{13}C]-, [4-^{13}C]-, and [3,4-$^{13}C_2$]DXP were synthesized enzymatically (Supporting Information [SI]).[11,12] HSQC spectra were recorded at 25 °C on a Varian INOVA-500 spectrometer equipped with a conventional $^{1}H\{^{13}C,^{15}N\}$ probe. Details of data acquisition and processing are provided in the SI.

First, the relative sensitivity of 1D ^{13}C NMR and 2D [^{13}C,^{1}H]-HSQC was assessed by acquiring spectra for a mixture of [3-^{13}C]- and [3,4-$^{13}C_2$]DXP. For the same measurement time, the *S*/*N* was 7-fold higher in the 2D spectra (Figure 2a). This sensitivity increase is predicted with Monte Carlo simulations to result in about a 5-fold increased precision in KIE measurements (SI).

Second, the 4-^{13}C KIE was measured in triplicate with a mixture of [3-^{13}C]- and [3,4-$^{13}C_2$]DXP as substrates for *Mt*DXR in the presence of NADPH and Mg^{2+} at pH 8.2. For each of the spectra recorded over the course of the reaction, 1D ^{13}C(f1) cross sections centered at δ(^{1}H-3) were added, revealing relative accumulation of ^{13}C at C-4 in the unreacted DXP (Figure 2b). Zero-filling in t_1 to 1024 complex points resulted in a high digital resolution (0.1–0.2 Hz) for accurate and precise integration of the peaks arising from the two isotopologues and the internal standard (not shown).[13,14] R/R_0 and F_1 were calculated from these integrals (Table S4 in SI), and fitting of eq 1 to the data (Figure 2c) yielded an average 4-^{13}C KIE of 1.0148(2) (uncertainty in last significant figure in parentheses; Table 1).[c]

Table 1. ^{13}C KIEs for *Mt*DXR

KIE	DXP pair	method	experimental KIE[a]
2-^{13}C	1-^{13}C and 1,2-$^{13}C_2$	2D HSQC	1.0034(3)
			1.0033(4)
			1.0027(4)
		ave	**1.0031(4)**
3-^{13}C	4-^{13}C and 3,4-$^{13}C_2$	2D HSQC	1.0291(12)
			1.0303(12)
			1.0316(11)
		ave	**1.0303(12)**
4-^{13}C	3-^{13}C and 3,4-$^{13}C_2$	2D HSQC	1.0150(6)
			1.0146(7)
			1.0148(8)
		ave	**1.0148(2)**
		1D ^{13}C	1.016(3)
			1.016(4)
			1.015(4)
		ave	**1.016(1)**

[a]KIEs were determined by fitting of eq 1 using nonlinear regression. Errors reported for individual experiments are errors of curve fitting, while those for averaged values are standard deviations from the triplicates.

The remarkably small experimental errors evidence the high precision of the 2D NMR-based measurements. To compare precision, the 4-^{13}C KIE was also measured in triplicate using the established 1D ^{13}C NMR method. Spectra were acquired on the same spectrometer and over the same total experiment time per spectrum as for the 2D spectra. The resulting data points (Figure 2d) are more scattered when compared with the 2D measure-

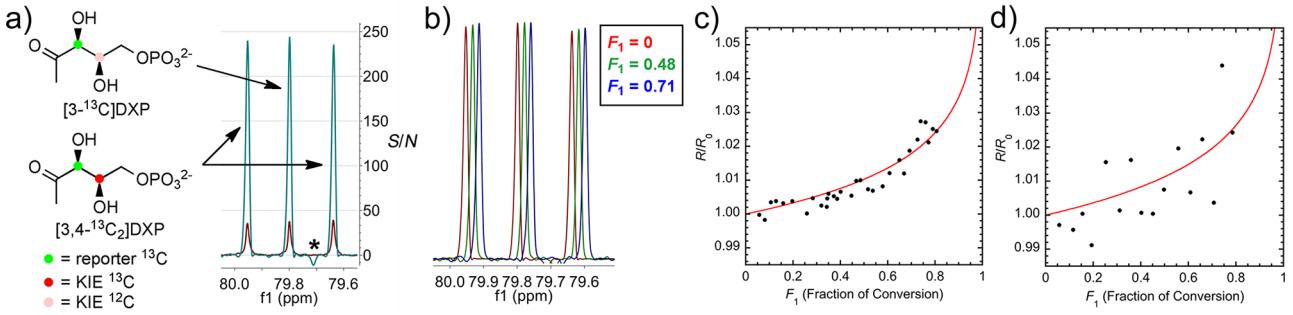


Figure 2. $^{13}C/^{12}C$ ratios from 2D [^{13}C,^{1}H]-HSQC and 1D ^{13}C NMR exemplified for the 4-^{13}C KIE. (a) The intrinsic sensitivity of such 1D (red) and 2D (blue) spectroscopy was evaluated from 500 MHz data acquired in 13 min for a sample containing [3-^{13}C]- and [3,4-$^{13}C_2$]DXP at a 1:2 mol ratio. The f1(^{13}C) cross section of the 2D spectrum was generated by adding twenty-six 1D f1(^{13}C) slices centered about δ(H-3) = 4.52 ppm (a small spectral artifact not affecting the measurement is marked with an asterisk). (b) Representative f1(^{13}C) cross sections at indicated fractions of conversion, F_1, after addition of *Mt*DXR. To illustrate the relative accumulation of the heavy isotopologue, the spectra are normalized to the intensity of the central singlet line that arises from the light isotopologue. Comparison of R/R_0 ratios versus F_1 measured using (c) 2D [^{13}C,^{1}H]-HSQC and (d) 1D ^{13}C NMR. The red curves were obtained by nonlinear least-squares fits of eq 1 to the data.

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ments (Figure 2c), yielding an average KIE of 1.016(1). In agreement with the Monte Carlo simulations, the experimental errors are each about 5-fold higher when compared with 2D (see SI for details). Nevertheless, the 4-^{13}C KIEs derived from 1D and 2D NMR are identical within the experimental errors, which provides evidence for the accuracy of the 2D method.

Third, to further validate the accuracy of the new approach, we measured in triplicate (SI) the 1-^{13}C KIE for *Leuconostoc mesenteroides* G6P dehydrogenase, which had been calculated to be 1.0165(7) by Hermes and co-workers[15] using isotope ratio MS. In excellent agreement, we obtained an average 1-^{13}C KIE of 1.0172(2).

Fourth, we measured the 2- and 3-^{13}C KIEs for *Mt*DXR (Table 1). The 3-^{13}C KIE of 1.0303(12), like the 4-^{13}C KIE, is consistent with a primary isotope effect associated with the C−C bond cleavage during the retroaldol step (Scheme 1). Distinct from the 4-^{13}C KIE, however, the nearly 2-fold larger 3-^{13}C KIE may reflect an additional primary effect that occurs during the reduction step, which has been established as partially rate limiting on k_{cat}/K_m.[16,17] This possibility may be addressed by the multiple isotope effect approach employed by Cleland and co-workers.[15,18] In contrast, the small 2-^{13}C KIE of only 1.0031(4) is consistent with a secondary KIE that results from conversion from the ketone to the enediol(ate) **1**, similar to observations for L-ribulose-5-phosphate 4-epimerase.[19] Hence, conversion of DXP to **3** (Scheme 1) is cleanly limited by the retroaldol step leading to **1** and **2**. Importantly, the small 2-^{13}C KIE provides further evidence against a [1,2]-sigmatropic rearrangement involving direct C-4 to C-2 migration, which would have resulted in a large primary KIE. The precisely measured, small 2-^{13}C KIE therefore supports the similar conclusion reached by Liu and co-workers[10] using ^{2}H KIEs with *Escherichia coli* DXR.

To conclude, the small experimental errors of 2D HSQC-based KIE measurements (between 0.02% and 0.12%; Table 1) are potentially surpassed only by those from isotope ratio MS.[20,21] Considering that (1) high-field NMR spectrometers for acquisition of 2D [^{13}C,^{1}H]-HSQC spectra are widely accessible and (2) no additional sample manipulation is required, the 2D approach presented here likely offers a more readily usable alternative for highly precise measurement of KIEs. This is particularly beneficial for measurement of small (e.g., secondary ^{13}C) KIEs.[22] Alternatively, one may choose to trade off the high precision exemplified in this study to acquire NMR data more rapidly or to use smaller sample quantities. Moreover, when compared with 1D ^{13}C NMR, the increased signal separation of 2D NMR is advantageous in cases where ^{13}C signals overlap, and the higher sensitivity of 2D [^{13}C,^{1}H]-HSQC may, in favorable cases, be sufficient to measure ^{13}C KIEs at natural abundance. A potential limitation of the 2D [^{13}C,^{1}H]-HSQC approach is its requirement of a ^{13}C−^{1}H moiety adjacent to the atom of interest. However, in most cases that have employed 1D NMR to measure ^{13}C KIEs, there is at most one carbon that fails to meet this condition.[22−29] It is also important to note that the 2D [^{13}C,^{1}H]-HSQC-based approach method is not limited to the measurement of ^{13}C KIEs. Provided an adjacent C−H bond exists to serve as a reporter, other KIEs (e.g., ^{2}H, ^{15}N, ^{18}O, and ^{37}Cl)[30,31] can be measured. Finally, the 2D spectra can potentially be acquired using sparse sampling approaches,[32,33] which allow recording of each 2D spectrum in less than a minute.

Taken together, the new HSQC-based method can be widely used for precise measurement of KIEs. In particular, measurements that are currently not feasible on NMR spectrometers equipped with conventional ^{13}C-detection probes can now be pursued on highest-field NMR spectrometers equipped with (cryogenic) probes designed for ^{1}H detection, thereby leveraging investments made for NMR-based structural biology. In turn, this promises to greatly enhance our understanding of transition states of chemical and enzymatic reactions.

■ ASSOCIATED CONTENT

Supporting Information

Preparation of [^{13}C]-DXPs, details for 1D ^{13}C and 2D [^{13}C,^{1}H]-HSQC NMR experiments and data analysis, tabulated and graphical data from KIE determinations, Monte Carlo simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

Notes

The authors declare no competing financial interest.

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■ ADDITIONAL NOTES

[a]The upfield shifts result from an isotope effect on the reporter ^{13}C chemical shift.[31]

[b]S/N in 2D [^{13}C,^{1}H]-HSQC relative to 1D ^{13}C NMR is proportional to $(\gamma_H/\gamma_C)^{5/2}$.

[c]The presence of ^{13}C at the reporter position exerts a minor effect on the commitments to catalysis; the observed KIEs are expected to differ negligibly from those with ^{12}C at this position.

■ REFERENCES

(1) Schramm, V. *J. Biol. Chem.* **2007**, *282*, 28297.

(2) Murkin, A. S.; Schramm, V., L. In *Drug Design: Structure- and Ligand-Based Approaches*, 1st ed.; Merz, K., Ringe, D., Reynolds, C., Eds.; Cambridge University Press: New York, 2010; p 215.

(3) Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; Wiley and Sons: New York, 1980.

(4) Chan, J.; Lewis, A. R.; Gilbert, M.; Karwaski, M. F.; Bennet, A. J. *Nat. Chem. Biol.* **2010**, *6*, 405.

(5) Ernst, R. R.; Bodenhausen, G.; Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*; Oxford University Press: London/New York, 1987.

(6) Kay, L. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663.

(7) Szyperski, T. *Q. Rev. Biophys.* **1998**, *31*, 41.

(8) Cavanagh, J.; Fairbrother, W. J.; Palmer, A. G.; Rance, M.; Skelton, N. J. *Protein NMR Spectroscopy*, 2nd ed.; Academic Press: New York, 2007.

(9) Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9879.

(10) Munos, J. W.; Pu, X.; Mansoorabadi, S. O.; Kim, H. J.; Liu, H.-W. *J. Am. Chem. Soc.* **2009**, *131*, 2048.

(11) Gao, W.; Raschke, M.; Alpermann, H.; Zenk, M. *Helv. Chim. Acta* **2003**, *86*, 3568.

(12) Hecht, S.; Wungsintaweekul, J.; Rohdich, F.; Kis, K.; Radykewicz, T.; Schuhr, C. A.; Eisenreich, W.; Richter, G.; Bacher, A. *J. Org. Chem.* **2001**, *66*, 7770.

(13) Herring, F. G.; Phillips, P. S. *J. Magn. Reson.* **1985**, *62*, 19.
(14) Malz, F.; Jancke, H. *J. Pharm. Biomed. Anal.* **2005**, *38*, 813.
(15) Hermes, J. D.; Roeske, C. A.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1982**, *21*, 5106.
(16) Argyrou, A.; Blanchard, J. S. *Biochemistry* **2004**, *43*, 4375.
(17) Liu, J.; Murkin, A. S. *Biochemistry* **2012**, *51*, 5307.
(18) Rendina, A. R.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 6257.
(19) Lee, L. V.; Vu, M. V.; Cleland, W. W. *Biochemistry* **2000**, *39*, 4808.
(20) Du, X.; Ferguson, K.; Gregory, R.; Sprang, S. R. *Anal. Biochem.* **2008**, *372*, 213.
(21) Paneth, P. In *Isotope Effects in Chemistry and Biology*; Kohen, A., Limbach, H.-H., Eds.; CRC Press: Boca Raton, 2006; p 875.
(22) Kelly, K. K.; Hirschi, J. S.; Singleton, D. A. *J. Am. Chem. Soc.* **2009**, *131*, 8382.
(23) Singleton, D. A.; Thomas, A. A. *J. Am. Chem. Soc.* **1995**, *117*, 9357.
(24) Brecker, L.; Kögl, M. F.; Tyl, C. E.; Kratzer, R.; Nidetzky, B. *Tetrahedron Lett.* **2006**, *47*, 4045.
(25) Singleton, D. A.; Szymanski, M. J. *J. Am. Chem. Soc.* **1999**, *121*, 9455.
(26) Meyer, M. P.; DelMonte, A. J.; Singleton, D. A. *J. Am. Chem. Soc.* **1999**, *121*, 10865.
(27) Gonzalez-James, O. M.; Zhang, X.; Datta, A.; Hrovat, D. A.; Borden, W. T.; Singleton, D. A. *J. Am. Chem. Soc.* **2010**, *132*, 12548.
(28) Nowlan, D. T.; Gregg, T. M.; Davies, H. M. L.; Singleton, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 15902.
(29) Lee, J. K.; Bain, A. D.; Berti, P. J. *J. Am. Chem. Soc.* **2004**, *126*, 3769.
(30) Aliev, A. E.; Harris, K. D. M. *Magn. Reson. Chem.* **1993**, *31*, 54.
(31) Hansen, P. E. *Prog. Nucl. Magn. Reson. Spectrosc.* **1988**, *20*, 207.
(32) Atreya, H. S.; Szyperski, T. *Methods Enzymol.* **2005**, *394*, 78.
(33) Hyberts, S. G.; Arthanari, H.; Wagner, G. *Top. Curr. Chem.* **2012**, *316*, 125.