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NMR study of ^{13}C-kinetic isotope effects at ^{13}C natural abundance to characterize oxidations and an enzyme-catalyzed reduction

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Abstract—^{13}C-kinetic isotope effects (KIEs) of four cinnamyl alcohol oxidations and a xylose reductase-catalyzed cinnamyl aldehyde reduction have been determined by ^{13}C NMR using competition reactions with reactants at natural ^{13}C-abundance. Differences in KIEs among oxidations indicate dissimilarities between the respective hydrogen transfers. Their mechanistic implications are discussed. A low primary KIE of the enzymatic reduction is consistent with a kinetically complex mechanism in which steps other than the chemical step of hydride transfer from NADH are slow.

1. Introduction

Kinetic isotope effects (KIEs) are a powerful tool to examine transition states and mechanistic details in a wide variety of reactions.[1–5] They indicate differences between vibrational surroundings of atoms in the ground and transition state of a reactant. So far, mainly hydrogen KIEs have been applied for that purpose, as the effect is most pronounced in case of hydrogen isotopes that possess a large relative weight difference. However, since small heavy-atoms also show measurable KIEs, carbon KIEs have also been used as a valuable tool for mechanistic studies.[5,6] Competition reactions between labeled and unlabeled reactants allow a NMR based simultaneous determination of ^{13}C-KIEs for carbon atoms in each position in a molecule, even at the low natural abundance of ^{13}C (1.108%).[6] Syntheses of isotopic labeled compounds can hence be avoided, and the method was applied to investigate ^{13}C-KIEs in certain chemical reactions.[7] Furthermore, it has been used to study an enzyme-catalyzed transformation.[5] We now present an application to investigate ^{13}C-KIEs in oxidations of cinnamyl alcohol **1** to cinnamyl aldehyde **2** using four different chemical oxidation methods. Furthermore, a xylose reductase-catalyzed reduction of **2** was studied.[8] These investigations allow a comparison of C–H bond breaking and hydrogen transfer as well as of the rate-limitation by the first irreversible steps in the mechanisms.[1,2]

2. Chemical oxidations

Manganese dioxide oxidation is well known to oxidize primary allylic alcohols to unsaturated aldehydes.[9,10] In this reaction, the hydroxyl anion binds coordinatively to hydrated $MnO(OH)_2$ on the catalysts surface. Then breaking of a C–H bond and hydrogen transfer to manganese oxide form an allyl-radical stabilized by the aromatic ring. An electron transfer leads to the final products, as shown in Figure 1a.[9,10] ^{13}C-KIEs of this cinnamyl alcohol **1** oxidation were determined for all carbon atoms (Fig. 1b). The KIE of C-5 has been taken as a reference (1.000) for all investigated oxidations, as this position is remote from the reaction center at C-1 and is only slightly influenced by secondary KIEs. All further secondary ^{13}C-KIEs of the MnO_2 oxidation are only slightly larger, while the primary ^{13}C-KIE shows a distinct higher value of 1.020(5)[11] (Fig. 1b). KIEs of C-2 and C-6 are determined together, as the shift difference of the signals is too small for separate integration.

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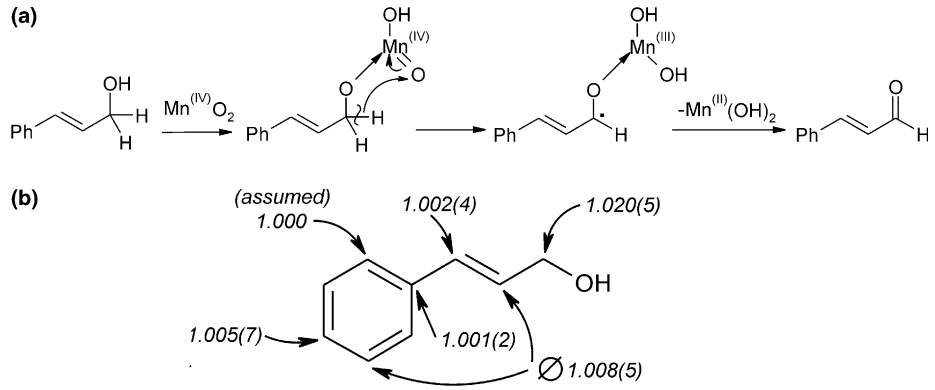

Figure 1. Proposed mechanism[10] (a) and ^{13}C-KIEs (b) of MnO_2 oxidation.[9,10]

Palladium(II)-catalyzed oxidations were only rarely used to oxidize primary allylic alcohols to aldehydes.[12,13] In the proposed mechanism of this reaction,[12] the hydroxyl anion coordinates to Pd(II) and the breaking of the C–H bond proceeds via hydride migration to Pd(II). As a result, the unsaturated aldehyde and a palladium hydride species are generated. Palladium is recycled by reductive elimination to Pd(0) and is oxidized to Pd(II) by molecular oxygen from the air (Fig. 2a). The ^{13}C-KIEs of atoms C-3 and C-7 are only negligibly larger, while the effect of C-4 is 1.006(4). The primary ^{13}C-KIE of C-1, however, is distinctly higher and shows a value of 1.018(3) (Fig. 2b).

The mechanism of the often applied Swern oxidation is well investigated.[14,15] An alkoxy anion and an 'activated DMSO' form a sulfonium salt, which is deprotonated to generate a sulfonium-ylide. Then a cyclic transition state is formed leading to the aldehyde and dimethylsulfide via a β-elimination. The C–H bond is heterolytically cleaved in the transition state and the proton is transferred to the ylide-structure, while the resulting carbonyl double bond is formed (Fig. 3a). ^{13}C-KIEs of atoms C-3 and of C-2 and C-6 are slightly below 1.000 and indicate a moderate inverse ^{13}C-KIE in these positions. The KIEs of C-4 and C-7 in the aromatic ring are slightly higher. The primary KIE has a value of 1.001(3) and is only insignificantly different from those of the reference KIE of C-5 (Fig. 3b).

Another often used method to oxidize primary alcohols to aldehydes is the Dess–Martin oxidation.[16,17] In this transformation, the alcohol reacts with triacetoxyperiodinane to form an alkoxydiacetoxyperiodinane that decomposes to the aldehyde and acetoxyiodinane (Fig. 4a). Two similar, but competitive mechanisms are discussed for the decomposition. One proceeds via a cyclic transition state with an intramolecular transfer of one proton from the alkoxide ligand to an acetate ligand (i).[17] In the alternative mechanism a proton is abstracted by a free acetoxy anion. The following electron transfer cascade expires in the detachment of another acetoxy anion (ii).[17] For the Dess–Martin reactions, we used freshly prepared triacetoxyperiodinane containing negligible amounts of acetoxy anions at the beginning of each transformation. This makes (i) the

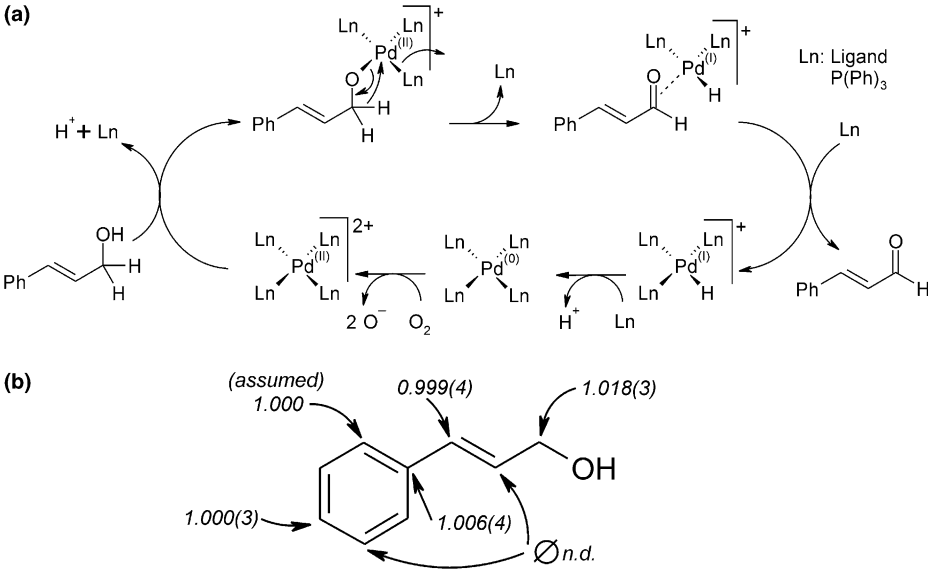

Figure 2. Proposed mechanism[13] (a) and ^{13}C-KIEs (b) of Pd(II)-catalyzed oxidation.[12,13]

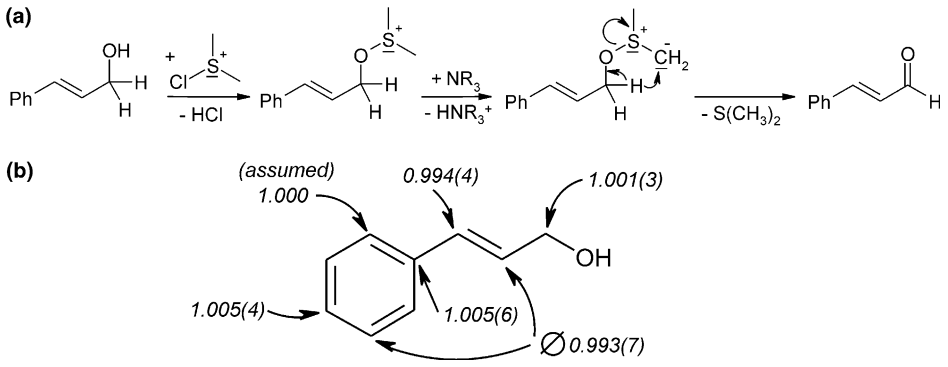

Figure 3. Mechanism[15] (a) and ^{13}C-KIEs (b) of Swern oxidation.[14,15]

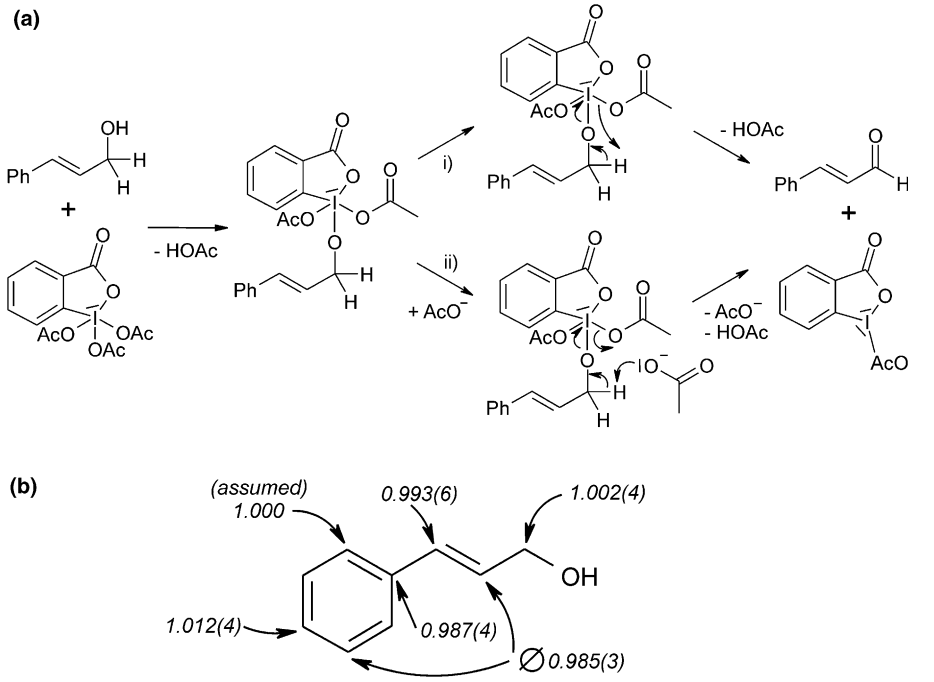

Figure 4. Two competitive mechanisms[17] (a) and ^{13}C-KIEs (b) of Dess–Martin oxidation.[16,17]

most likely mechanism (Fig. 4a). However, mechanism (ii) can occur parallelly, as acetoxy anions are generated during the reaction. The primary ^{13}C-KIE is 1.002(4). All secondary KIEs of atoms C-2 and C-6, C-3, and C-4 are moderately below the one of C-5 indicating slight inverse KIEs and the one of C-7 is exceptionally high (Fig. 4b).

3. Xylose reductase-catalyzed reduction

The reduction of **2** to **1** by an aldo–keto reductase from the yeast *Candida tenuis* is shown in Figure 5a.[8,18] This enzymatic reaction proceeds through a hydride transfer from C-4 of protein-bound NADH to the carbonyl group of **2**, whereby the proton that is required to produce the alcohol is provided from the side chain of tyrosine 51. The primary ^{13}C-KIE of this reaction is slightly higher (1.007(5)) than the KIE of atom C-6, which was taken as a reference (Fig. 5b). KIEs of the other atoms do not markedly differ from the primary one, except the one of C-4 (0.997(8)), which is in the range of the reference KIE of C-6. All ^{13}C-KIEs show slightly larger standard deviations than the corresponding KIEs of the chemical oxidations. This problem is likely caused by the low solubility of **1** and **2** in water and the consequential comparatively quite small amount of re-extracted starting material used for characterizing the enzymatic reaction. However it is not an intrinsic limitation of the analytical method to investigate biocatalyzed reactions.

4. Mechanistic interpretation of KIEs

^{13}C-KIEs clearly reveal mechanistic differences among the four oxidation methods studied. Primary ^{13}C-KIEs of the Pd(II)-catalyzed oxidation and of the MnO_2 oxidation are quite similar (Figs. 1b and 2b), although the proposed mechanisms indicate heterolytic and

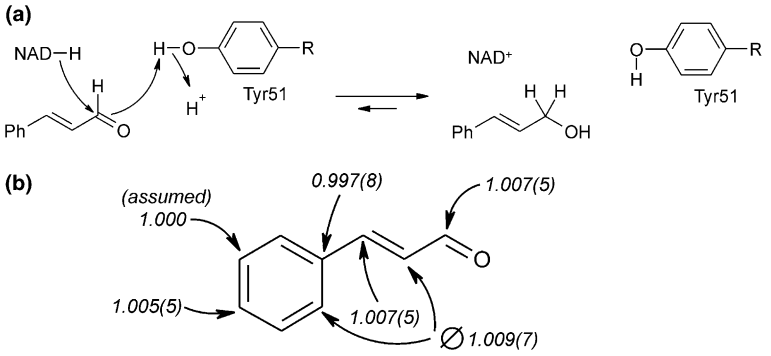

Figure 5. Simplified mechanism[18] (a) and ^{13}C-KIEs (b) of xylose reductase-catalyzed reduction.[8,18,25]

homolytic C–H bond cleavages leading to a hydride and a hydrogen atom, respectively. Several other oxidations by metal ions or metal oxides like Cu(I), CrO_3, and MnO_4^- have earlier been reported to possess primary ^{13}C-KIEs, which are also in the range of 1.020.[19–23] The KIEs of CrO_3 oxidation of acetic acid[20,24] and of MnO_4^- oxidation of propionate[21] have been intensively investigated, and indicate the C–H bond cleavages to be the irreversible and rate-limiting steps in the respective mechanisms.[20,21] These mechanisms are similar to those of the MnO_2 oxidation and of the Pd(II)-catalyzed oxidation. Hence, the quite high primary ^{13}C-KIE in these two cinnamyl alcohol **1** oxidations can also be used to indicate C–H bond breaking and hydrogen transfer to be the slow, irreversible, and rate-determining steps leading to energy rich substrate-like transition states.

Reasonable low primary ^{13}C-KIEs in Swern and Dess–Martin oxidations (Figs. 3b and 4b), however, indicate that the initial C–H bond breakings and proton transfers are not the irreversible steps in these mechanisms, which control the rate. Rather, the resulting protons migrate close to the anionic acceptors and promote the cleavages of the poor leaving groups, respectively. The C–H bond breakings, therefore, do not directly lead to energy rich transition states and are not rate-limiting.

Not all hydride transfers necessarily control the rate in catalytic oxidations and reductions, as described in the past for several enzymatic conversions in which kinetic complexity led to partial or complete masking of the KIEs. Previous primary deuterium KIE studies of the aldo–keto reductase from the yeast *C. tenuis* have revealed that the 'chemical' reaction steps are only partly rate-limiting during reduction of xylose and aromatic aldehydes.[8] The ^{13}C-KIEs of the enzymatic reduction of **2** to **1** (Fig. 5b) are in good agreement with this notion, as they support a mechanism in which slow steps occur outside the catalytic sequence involving the hydride transfer.[25] The KIEs reported here are essentially effects on the catalytic efficiency (V/K) and can be compared to KIEs on V/K for other NAD(P)H-dependent dehydrogenases in the literature.[26] However, they can not be considered for all substrates being transformed by the investigated aldo–keto reductase. Some recently reported primary deuterium KIEs of other pyridine nucleotide dependent enzymes indicate that each reaction possesses its own unique KIEs.[27,28] The results thus show that the NMR analysis of ^{13}C-KIEs at natural ^{13}C-abundance will be conducive to examine dehydrogenase-catalyzed reactions.[26]

5. Secondary ^{13}C-KIEs

Secondary ^{13}C-KIEs of the investigated reactions are of particular interest, because double bond and aromatic ring are conjugated with the p-orbital of the reaction center. Separate α-^{13}C-KIEs could not be determined by the applied method, as the ^{13}C-signals of these nuclei are overlapped by other signals in both starting materials. The β-^{13}C-KIEs of **1** and **2**, however, are lowest in all four oxidations and only slightly higher in the reduction. They indicate vibrational surroundings in these positions to influence the formation of the transition states. This effect is likely caused by the conjugated system, which includes the reaction center as well as the carbon atoms in the α- and β-positions. It is probably similar to the deuterium KIE effects, which cause small or inverse α- and β-^{2}H-KIEs by hyperconjugation in reactions with transitions between sp^2- and sp^3-centers.[29]

Further on, in all reactions KIEs of some aromatic carbons are clearly higher than the reference KIE, in particular those of the *para*-positions. This indicates vibrational surroundings of atoms in the conjugated system to be in correlation to the formation of the energy rich transition states, even when they are more than three bonds away from the reaction center. The preferred acceptance of conjugated aromatic substrates by some of the oxidation reagents and by the aldo–keto reductase is probably promoted by the influences from the vibrational behavior of atoms in the conjugated aromatic systems.

6. Conclusions

To summarize, ^{13}C-KIEs determined by NMR at ^{13}C-natural abundance give an insight into the mechanistic details of C–H bond breaking and hydrogen transfer of alcohol oxidations and an aldehyde reduction. Oxidations with an intermittent alkoxy bound poor leaving group proceed via a fast proton transfer that is not

rate-determining. In oxidations, which are induced by the reduction of a metal ion, a hydrogen atom or a hydride migrates to the metal ion in the first irreversible and rate-limiting step. The mechanism of aldo–keto reductase-catalyzed reductions depends on a hydride transfer from NADH to the carbonyl group, which is, however, not the main step controlling the transformation rate.

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Supplementary data

Detailed experimental procedures as well as ^{1}H and ^{13}C NMR spectroscopic data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.03.194.

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