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A simple method for the precise and simultaneous determination of primary and multiple secondary kinetic deuterium isotope effects in organic reactions at natural abundance

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ABSTRACT: A method for the determination of kinetic isotope effects (KIEs) in organic reactions using natural abundance deuterium NMR is proposed. The method consists of the determination of the site-specific H/D isotopic ratios of the starting reactant and the product in a reaction run under pseudo-first-order kinetic conditions. Using simple and easy experimental procedures, primary and/or multiple secondary KIEs can be measured simultaneously with high precision. The application of this approach to different types of reaction is described. The primary and α-secondary KIEs for C—H(D) bond breaking of —CDH— can be measured separately. The uncertainty in the determination of small secondary KIEs can reach less than 1%. The results show that some remote secondary KIEs are not negligible. The advantages and limitations of the method are discussed. One of its merits is that the method can even be used in complex situations when there are branch reactions and when the reaction is reversible. The performance depends essentially on the signal separation in the NMR spectra. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: reaction kinetics; isotope effects; natural abundance; deuterium; NMR

INTRODUCTION

It has been well established that chemical kinetic isotope effects (KIEs) can be determined at natural abundance using ^{2}H NMR and several experimental methods have been proposed.[1–6] Each of these methods has its advantages and disadvantages but none of them is suitable for all applications.[7] Thus, it is necessary to develop more experimental approaches in order to facilitate the study of KIEs at natural abundance. Today, high-resolution NMR spectrometers are available in many laboratories and current NMR data acquisition and manipulation methods allow us to obtain more accurate quantitative ^{2}H NMR results. It should be noted that, in spite of the very low natural abundance of deuterium (0.015%), the quantitative analysis of ^{2}H at natural abundance by NMR can be more precise than that of ^{1}H by NMR. This is because in ^{2}H NMR only monodeuterated molecules whose signal is a singlet are analysed, whereas in ^{1}H NMR the signal of a non-equivalent hydrogen is often a multiplet for which the precise measurement of the surface area under the peak is more difficult. This background favours the development and the application of natural abundance ^{2}H NMR in KIE studies. Here we present a method based on competition under pseudo-first-order conditions for the measurement of KIEs. The pseudo-first-order kinetic method has been frequently applied to the determination of KIEs using isotopically enriched substrates. Our interest is to determine KIEs at natural abundance by this method. The experimental procedure required is very simple and it has been used in the determination of the primary KIE of the hydrolysis of Grignard reagents.[4] In this paper, it is shown that this method can be applied to a wide range of reactions and that not only can primary and different secondary KIEs be determined simultaneously but also that very high precision can be obtained.

METHOD

In a chemical reaction, there is competition between unlabelled and naturally monodeuterated molecules. As there are a number of isotopomers monodeuterated at different sites of the molecule, several competitions may

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occur simultaneously:

$$S + HR \xrightarrow{k_H} HP$$

$$S + D_iR \xrightarrow{k_{D_i}} D_iP$$

In the equation, the subscript i indicates site i of the reactant R which reacts with the substrate S in forming a product P in which the hydrogens of R are found; at site i there are p_i equivalent hydrogen atoms, for example, for methyl, CH_3, $p_i = 3$. For S, only the unlabelled molecules are considered in the above reactions. At natural abundance, the reaction between monodeuterated S and monodeuterated R can be disregarded. HR refers to the unlabelled molecules of R. Its rate constant in the reaction forming HP is k_H:

$$d[HP]/dt = k_H[S][HR] \quad (1)$$

D_iP is a monodeuterated molecule formed from a monodeuterated reactant R, D_iR. The corresponding rate constant is k_{D_i}:

$$d[D_iP]/dt = k_{D_i}[S][D_iR] \quad (2)$$

From Eqns (1) and (2), we have

$$d[HP]/d[D_iP] = (k_H/k_{D_i})[HR]/[D_iR] \quad (3)$$

In the experiment, the reactant R is always introduced in large excess. Thus, during the reaction, $[HR]/[D_iR] \approx$ constant (i.e. pseudo-first-order reaction kinetic conditions are maintained). Integration of Eqn. (3) gives

$$[HP]/[D_iP] = (k_H/k_{D_i})[HR]/[D_iR] \quad (4)$$

The specific hydrogen isotope ratio (in ppm) of site i is defined as $(D/H)_i = N_{D_i}/(p_iN_H)$[8] where N_{D_i} is the number of isotopomers monodeuterated at site i and N_H is the number of the unlabelled species. In the cases where site i is not the reaction centre and the stoichiometric number of hydrogens at position i, p_i, does not change when HR is converted to HP, such as in the case of remote secondary KIEs, both sides of Eqn. (4) are multiplied by p_i and we have

$$p_i[HP]/[D_iP] = (k_H/k_{D_i})p_i[HR]/[D_iR] \quad (5)$$

Since the specific isotopic ratio of site i in R $(D/H)_{i,R} = [D_iR]/(p_i[HR])$ and that in P $(D/H)_{i,P} = [D_iP]/(p_i[HP])$,

$$k_H/k_{D_i} = (D/H)_{i,R}/(D/H)_{i,P} \quad (6)$$

The KIE(s) can thus be determined through the analysis of site-specific isotopic ratios of the reactant (R) and the product (P). In the experiment, the complete conversion of S is not necessary if P can be isolated easily from the reaction mixture. $(D/H)_{i,R}$ and $(D/H)_{i,P}$ can be determined with very high precision using the SNIF NMR method.[8,9] The error of the measurement can be estimated by the following equation:

$$\Delta(k_H/k_{D_i}) = k_H/k_{D_i}\sqrt{\left[\frac{\Delta(D/H)_{i,R}}{(D/H)_{i,R}}\right]^2 + \left[\frac{\Delta(D/H)_{i,P}}{(D/H)_{i,P}}\right]^2} \quad (7)$$

Although Eqn. (6) is obtained for the cases in which p_i does not change from HR to HP, it is also valid for cases where i is the reaction centre and the number of non-equivalent hydrogens is different in HR and in HP. This will be demonstrated below in a concrete example in which, for the reaction centre i, the primary effect and α-secondary effect are simultaneously determined using Eqn. (6). Generally, if $H(D)_i$ is involved in bond breaking, k_H/k_{D_i} is a primary effect, KIE(I). Otherwise, it is a secondary effect, KIE(II).

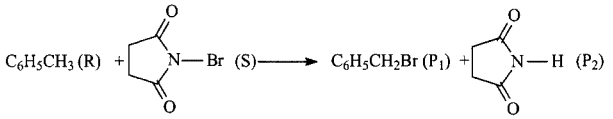
So long as R is in large excess, the (secondary) KIEs of the monodeuterated isotopomers of S cannot be determined. However, by repeating the experiment with S in large excess, similar pseudo-first-order conditions are established in which S now behaves as R (the reactant in large excess) and R as S. The KIEs can therefore be calculated using Eqn. (6).

RESULTS AND DISCUSSION

KIE(I) and KIE(II)s of bromination of alkylbenzenes

Two brominating agents, *N*-bromosuccinimide (NBS) and bromine, were used in the bromination of toluene:

$$C_6H_5CH_3(R) + Br_2(S) \rightarrow C_6H_5CH_2Br(P_1) + HBr(P_2)$$



Values of p_i for the different sites of the phenyl ring remain the same before and after the reaction. The reaction centre is the methyl of toluene, which is converted to a methylene in the product, leading to a change of p_i. In order to demonstrate the validity of Eqn. (6) in this example, we should consider the following competitive reactions:

$$Br_2(NBS) + PhCH_3 \xrightarrow{3k_H} PhCH_2Br + HBr(NH \text{ of succinimide})$$

$$Br_2(NBS) + PhCH_2D \xrightarrow{2k_{\alpha-D}} PhCHDBr + HBr(NH \text{ of succinimide})$$

$$Br_2(NBS) + PhCH_2D \xrightarrow{k_D} PhCH_2Br + DBr(ND \text{ of succinimide})$$

Table 1. Determination of KIE(I) and KIE(II)s in toluene bromination[a]

Bromination with Br_2 (50 °C)		$(D/H)_{Ph}$ (ppm)	$(D/H)_{CH_3}$ (ppm)	$(D/H)_{CH_2Br}$ (ppm)	$(D/H)_{NH(HB_R)}$ (ppm)
Expt 1	Starting toluene	155.6 (0.7)	122.4 (0.4)		
Expt 1	Benzyl bromide	149.1 (0.7)		132.4 (0.8)	
Expt 1	HBr–pyridine				91.1 (4.2)
Expt 2	Starting toluene	153.0 (0.3)	123.8 (0.3)		
Expt 2	Benzyl bromide	150.0 (0.8)		134.1 (0.4)	
					NH
Expt 1	KIE(I)				1.3(0.1)
		Phenyl	$CH_3(\alpha)$		
Expt 1	KIE(II)	1.044 (0.007)	0.924 (0.006)		
Expt 2	KIE(II)	1.020 (0.006)	0.923 (0.004)		

Bromination with NBS (110 °C)		$(D/H)_{Ph}$ (ppm)	$(D/H)_{CH_3}$ (ppm)	$(D/H)_{CH_2Br}$ (ppm)	$(D/H)_{NH(HBr)}$ (ppm)
	Starting toluene	155.6 (0.7)	122.4 (0.4)		
	Benzyl bromide	144.9 (0.8)		133.2 (0.9)	
	Succinimide				36.8 (1.0)
					NH
	KIE(I)				3.33 (0.09)
	KIE(II)	Phenyl	$CH_3(\alpha)$		
		1.074 (0.008)	0.919 (0.007)		

[a] The standard deviation is given in parentheses.

The rate equations for these reactions can be written, respectively, as

$$d[HBr(NH\ of\ succinimide)]/dt = d[PhCH_2Br]/dt = 3k_H[PhCH_3][Br_2(NBS)] \quad (8)$$

$$d[PhCHDBr]/dt = 2k_{\alpha-D}[PhCH_2D][Br_2(NBS)] \quad (9)$$

$$d[DBr(ND\ of\ succinimide)]/dt = k_D[PhCH_2D][Br_2(NBS)] \quad (10)$$

In Eqn. (8), the rates of formation of HBr (or succinimide) and $PhCH_2Br$ do not include those produced in the second and third of the above three reactions because they are involved in reactions with naturally deuterated isotopomers and can be disregarded.

Equations (8) and (10) give

$$\frac{d[HBr(NH\ of\ succinimide)]}{d[DBr(ND\ of\ succinimide)]} = \frac{3k_H[PhCH_3]}{k_D[PhCH_2D]} \quad (11)$$

and from Eqns (8) and (9) we obtain

$$\frac{d[PhCH_2Br]}{d[PhCHDBr]} = \frac{3k_H[PhCH_3]}{2k_{\alpha-D}[PhCH_2D]} \quad (12)$$

After integration, the $KIE(I) = k_H/k_D = (D/H)_{CH_3}/(D/H)_{HBr\ (NH\ of\ succinimide)}$ and the α-$KIE(II) = k_H/k_{\alpha\text{-}D} = (D/H)_{CH_3}/(D/H)_{CH_2Br}$. The validity of Eqn. (6) is justified. Accordingly, the calculation of the secondary KIEs is based on the analysis of toluene (R) and benzyl bromide (P_1) while for the calculation of the KIE(I), an additional analysis of P_2 (the succinimide or HBr which was converted to pyridinium bromide) is necessary. The results are summarized in Table 1.

The uncertainty for the KIE(I) values is larger than that for the KIE(II) values. This is related to the error of measuring the $(D/H)_{NH}$. Because the ^2H NMR (Fig. 1) signal of ND(or ND^+) is broad and of low intensity , the signal-to-noise ratio is smaller. Owing to the bad separation of the *o*-, *p*- and *m*-phenyl deuterium signals of the spectra of both reagent and product, only one average ring hydrogen KIE(II) has been obtained. We carried out a second kinetic experiment with bromine for which only KIE(II)s were determined and obtained the same KIE(II) values within standard deviations. The KIE(I) value for NBS bromination is of the same order as previously reported values: 3.59–6.4.[2,10]. The difference may be attributed to the difference in reaction conditions such as solvent, temperature, etc. The solvent under our working conditions is always the reactant in excess. The KIE(I) in the reaction with bromine is smaller than some published data and close to that of chlorination.[10a] This may also be related to the different experimental conditions used. It is worth noting that an exchange between HBr or HCl and the phenyl hydrogens may occur under certain conditions. It would be useful to

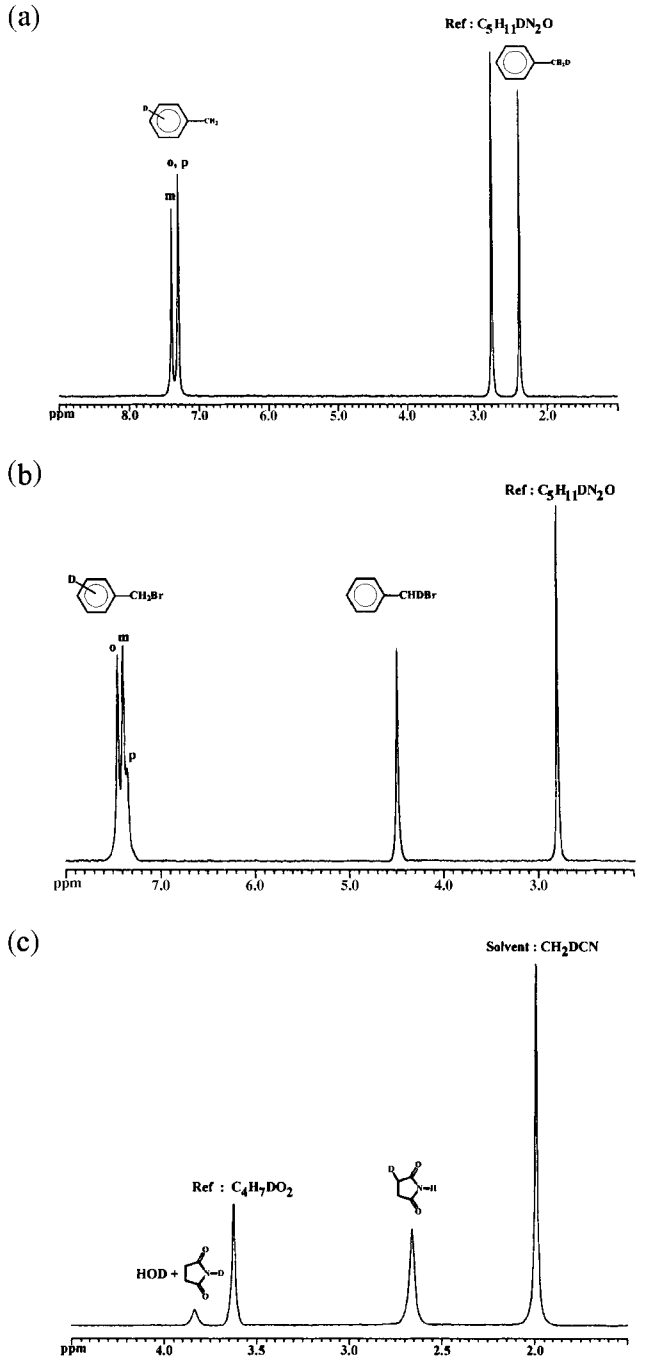


Figure 1. Natural abundance ^{2}H NMR spectra of the reactant and products of the reaction of bromination of toluene by NBS. (a) Reactant R (toluene); (b) product P_1 (benzyl bromide); (c) product P_2 (succinimide). In the spectra, each signal corresponds to a naturally monodeuterated isotopomer whose number is proportional to the surface area of the signal

estimate its influence on the KIE measurement. The α-KIE(II) value obtained in this work is comparable to that [0.85 (±0.10)] determined by natural abundance ^{2}H NMR with another method[2] in that both measurements find an inverse effect. However, the precision in the present work is much higher.

KIE(I) and KIE(II)s of Friedel–Crafts reactions

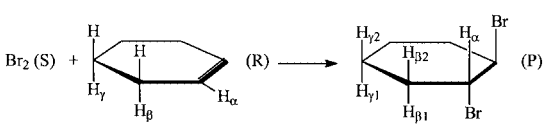
The acetylation of benzene catalyzed by $AlCl_3$ was studied:

$$C_6H_6(R) + CH_3COCl(S) \xrightarrow{AlCl_3} C_6H_5COCH_3(P_1) + HCl(P_2)$$

Because one of the six benzene hydrogens is in the reaction centre, two products P_1 and P_2 should be analysed for the calculation of KIE(I) and KIE(II). HCl (P_2) was converted to pyridinium chloride for ^{2}H NMR measurement of KIE(I). Since the signals of acetophenone are well separated, all KIE(II)s were also obtained (Table 2). The small normal value for KIE(I) is in good agreement with determinations made for other Friedel–Craft reactions.[11] The three ring hydrogen positions have different KIE(II)s of which the error of measurement is not more than 1%. The *o*-H shows no KIE whereas the *m*-H has a marked inverse effect and the *p*-H has a small normal effect. A major advantage of using natural abundance ^{2}H NMR for the determination of KIE is that multiple KIE(II)s can be determined simultaneously. For deuterium KIE(II)s, most studies focus on α- and β-KIE(II)s. According to the conventional view, α-KIE(II) is related to the changes in the C_α–H(D) out-of-plane bending vibrations when the reactant is converted into the transition state while β-KIE(II) is primarily a result of hyperconjugation.[7] Generally, when the force constants for the bonds to isotopic nuclei decrease on going from reactant to transition state in the rate-determining step, the KIE is normal; otherwise it is inverse. Several studies have shown that many remote KIE(II)s are not negligible and that the KIE(II)s of different sites in a molecule in a reaction are often very different: some may be normal whereas others are inverse or negligible. The mechanistic significance of these data has not yet been well studied. By combining these data with *ab initio* theoretical calculations, more details of the reaction mechanism could be revealed. The aim of this work is to present the methodology and the mechanistic significance of the KIE values will not be discussed in detail.

KIE(II)s of bromination of olefins

KIE(II)s for the bromination of cyclohexene:



were determined. The results are summarized in Table 3.

In this reaction there is no C—H bond breaking, but the number of non-equivalent hydrogens increases during the reaction. Precise KIE(II) values are obtained in the

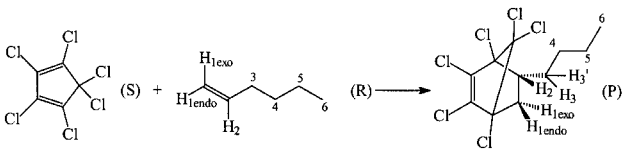
Table 2. Determination of KIE(I) and KIE(II)s in the acetylation of benzene (50 °C)

	$(D/H)_{ring}$ (ppm)	$(D/H)_{o\text{-}H}$ (ppm)	$(D/H)_{m\text{-}H}$ (ppm)	$(D/H)_{p\text{-}H}$ (ppm)	$(D/H)_{NH(HCl)}$ (ppm)
Starting benzene	138.5 (0.5)				
Acetophenone		138.6 (0.5)	141.3 (0.5)	136.8 (0.9)	
HCl–pyridine					103.2 (1.4)
KIE(I)					NH 1.34 (0.02)
KIE(II)		*o*- 0.999 (0.005)	*m*- 0.980 (0.005)	*p*- 1.012 (0.008)	

experiments. Marked inverse effects are observed at α, β_1 and γ_2 positions whereas there is no effect at β_2 and γ_1 sites. The inverse α-KIE(II) seems to indicate an sp^3-like transition state during the formation of bromonium ion.

KIE(II)s of a Diels–Alder reaction

The reaction studied is that between perchlorocyclopentadiene and hex-1-ene:



The results are given in Table 4.

The reaction is very slow under the experimental conditions. We took advantage of this to check the reaction kinetics and confirmed that the reaction is of first order for the substrate (S). The rate constant is 0.011 h^{-1} for the unlabelled molecules. In order to facilitate the purification of the product (it is difficult to separate the unreacted perchlorocyclopentadiene and the addition product), the reaction was not stopped until ~100% conversion was reached. Only one *endo* addition product was obtained: 5-butyl-1,2,3,4,7,7-hexachloro-bicyclo-[2.2.1]hept-2-ene (**1**).[12] In the NMR spectrum, there are more overlapping signals for **1** than for hex-1-ene. Since in the ^2H NMR spectrum of **1**, the signals of D_{1exo} and D_3 as well as those of $D_{3'}$ and D_6 cannot be separated, not all the site-specific KIE(II)s can be obtained. In addition, for the C-4 and C-5 hydrogens only an average value can be determined. Owing to incomplete signal separation, the precision of $(D/H)_i$ of some sites of the two molecules is not very good. Even so, the maximum error is only 1.9%. This example shows that the performance of the method

Table 3. Determination of KIE(II)s of cyclohexene bromination (20 °C)

Starting cyclohexene	$(D/H)_\alpha$ (ppm) 123.0 (0.7)	$(D/H)_\beta$ (ppm) 133.6 (0.8)		$(D/H)_\gamma$ (ppm) 126.6 (0.9)	
(*E*)-1,2-Dibromocyclohexane	$(D/H)_\alpha$ (ppm) 125.7 (0.4)	$(D/H)_{\beta1}$ (ppm) 138.6 (0.3)	$(D/H)_{\beta2}$ (ppm) 133.4 (0.5)	$(D/H)_{\gamma1}$ (ppm) 126.6 (0.7)	$(D/H)_{\gamma2}$ (ppm) 129.4 (0.7)
KIE(II)	α 0.979 (0.006)	β_1 0.964 (0.006)	β_2 1.001 (0.007)	γ_1 1.000 (0.009)	γ_2 0.978 (0.009)

Table 4. Determination of KIE(II)s in the Diels–Alder reaction between perchlorocyclopentadiene and hex-1-ene (65 °C)

Starting hex-1-ene	$(D/H)_{H_{1endo}}$ (ppm) 132.4 (1.9)	$(D/H)_{H_{1exo}}$ (ppm) 130.5 (0.5)	$(D/H)_{H_2}$ (ppm) 153.6 (1.4)	$(D/H)_{H_3}$ (ppm) 136.0 (1.1)	$(D/H)_{H_4,H_5}$ (ppm) 145.0 (0.9)	$(D/H)_{H_6}$ (ppm) 114.4 (0.6)
Product (**1**)	$(D/H)_{H_{1endo}}$ (ppm) 122.3 (2.3)	$(D/H)_{H_{1exo},H_3}$ (ppm) 137.6 (0.6)	$(D/H)_{H_2}$ (ppm) 160.1 (2.1)		$(D/H)_{H_4,H_5}$ (ppm) 169.5 (1.9)	$(D/H)_{H_{3'},H_6}$ (ppm) 126.8 (1.3)
KIE(II)	H_{1endo} 1.082 (0.026)		H_2 0.959(0.015)		H_4, H_5 0.855(0.011)	

Table 5. Determination of KIE(II)s in esterification (EtOH–PhCOCl, 78 °C; PrOH–PhCOCl, 97 °C)

Experiment No.		$(D/H)_{CH_2}$ (ppm)		$(D/H)_{CH_3}$ (ppm)
1	Starting ethanol	141.4 (0.8)		124.0 (0.3)
1	Ethyl ester	141.8 (0.7)		123.2 (0.3)
2	Starting ethanol	138.2 (1.4)		122.3 (0.9)
2	ethyl ester	141.2 (0.5)		121.5 (0.5)
		CH_2		CH_3
1	KIE(II) ethyl	0.997 (0.007)		1.006 (0.003)
2	KIE(II) ethyl	0.979 (0.010)		1.007 (0.008)
		$(D/H)_{\alpha\text{-}CH_2}$ (ppm)	$(D/H)_{\beta\text{-}CH_2}$ (ppm)	$(D/H)CH_3$ (ppm)
1	Starting ethanol	110.8 (0.7)	141.5(0.7)	129.4 (0.6)
1	Propyl ester	110.3 (0.6)	141.2(0.3)	130.1 (0.5)
2	Starting propanol	114.7 (0.6)	135.4 (0.3)	128.1 (0.7)
2	Propyl ester	115.6 (0.6)	136.9 (0.8)	130.6 (0.3)
		α-CH_2	β-CH_2	CH_3
1	KIE(II) propyl	1.005 (0.008)	1.002 (0.005)	0.995 (0.006)
2	KIE(II) propyl	0.992 (0.007)	0.989 (0.006)	0.980 (0.006)

depends critically on the resolution of the NMR signal. Nevertheless, significant KIE(II)s can be observed even taking into account this level of uncertainty. A theoretical calculation is necessary for the interpretation of the effects.

KIE(II) of esterification

The following esterifications were studied:

$$\mathrm{EtOH(R) + PhCOCl(S) \rightarrow PhCO_2Et(P) + HCl}$$
$$\mathrm{PrOH(R) + PhCOCl(S) \rightarrow PhCO_2Pr(P) + HCl}$$

Only the KIE(II)s of the alcohol moiety were determined (Table 5).

Two kinetic experiments were carried out and the results are in good agreement. Although the precision of the KIE(II) values is very high, the measured isotopic effects are negligible. In each experiment, the standard deviation reflects mainly the precision of the NMR measurement. However, the conditions of reaction and of product isolation and purification must be rigorously controlled. Notably, isotopic fractionation introduced during product isolation must be avoided essentially by minimizing losses during working. Neglecting these precautions will distort the KIE results. The KIE(I) of O—H bond breaking cannot be determined because the hydrogen of the HCl formed in the reaction dissolves in the large quantity of alcohol present, with which it is in rapid exchange. Thus, the precise measurement of its (D/H) value is not possible. The KIE(II)s of the phenyl moiety could also be studied by using an excess quantity of benzoyl chloride in the reaction. The experiment was not done in this work because the effects are expected to be very small, as shown for the alkyl moiety.

CONCLUSIONS

The above examples represent different cases in which the ^{2}H NMR method has been applied to the determination of primary and secondary KIE values at natural abundance. These examples illustrate effectively that the use of this method has many advantages: (1) The determination of fractional conversion of reactants[5,6] is not necessary and this simplifies the experimental procedure by avoiding the error in its determination. (2) The precision is generally high as, in most cases, the site-specific isotope ratios $(D/H)_i$ of organic compounds are of the order of 150 ppm (average natural abundance) and the NMR precision can generally reach $\Delta(D/H)_i \leq 0.5$ ppm. Thus, the error of small KIE values should not be larger than 1% according to Eqn. (7). As is shown by the results, the method is very suitable for the study of small KIE(II)s. (3) The KIE(II) for multiple sites can be determined simultaneously provided the resonances are sufficiently well resolved. (4) Since in the NMR measurement an external reference is used, no internal reference is necessary. The internal reference method relies on using a remote deuterium in the reacting molecule assumed to have no KIE.[2,6] However, the use of an internal reference must be treated cautiously. We show here that, in a number of the reactions studied, 'remote' hydrogens show significant KIE(II)s. The use of an external reference avoids the risk of choosing an internal reference which may have a non-negligible KIE(II). (5) As the measurement of KIEs is based on the analysis of

the reaction product (this corresponds to following the product formation kinetics), more KIE values can be obtained than for intermolecular competition method[6] based on analysis of the starting reagent (this corresponds to following the kinetics of disappearance of the reactant). This is particularly useful when the number of non-equivalent hydrogens increases after the reaction as, for example, in the acetylation of benzene. The intermolecular competition method cannot be used either for the KIE measurement of this reaction or for the determination of the KIE(I) and α-KIE(II) in toluene bromination. (6) The other methods[1,5,6] can only be used in 'clean' and irreversible reactions. The present method is still applicable even when there are branch reactions between S and R. If the products of the branch reactions can be isolated and analysed, the KIEs of these reactions can also be calculated. (7) In addition, it can be applied in some complicated situations such as reversible reactions. Since at the early stage the backward reaction can be disregarded, the KIEs of the forward reaction can be determined by quenching the reaction at this stage. In this case, the initial quantities of both reactants can be in stoichiometric proportion and their conversion fraction should not exceed 5% to ensure a 20-fold excess. In this situation, the KIEs of the monodeuterated isotopomers of both R and S can be calculated simultaneously from $(D/H)_i$ of R, S and P:

$$\text{for R: } k_H/k_{D_i} = (D/H)_{i,R}/(D/H)_{i,P} \qquad (13)$$

$$\text{for S: } k_H/k_{D_i} = (D/H)_{i,S}/(D/H)_{i,P} \qquad (14)$$

A critical parameter in determining $(D/H)_i$ values is the precision with which these signal surface areas are calculated. For all the quantitative NMR measurements reported here the signal surfaces were always calculated by using a curve-fitting program in which the analysis involves automatic integrated management of all experimental parameters, including the phases of the individual resonances.[13] The use of manual direct integration from spectra for the calculation of signal surface areas should be avoided. As the NMR integration depends on many factors, such as signal-to-noise ratio, lineshape, integration area, phase, baseline and integration correction, the results from manual integration may be sensitive to operators.[14] When using the automatic curve-fitting program, all operators obtain the same results at optimal curve fitting and this ensures the objectivity of the results.

In our work, only deuterium KIEs are reported. In principle, isotope effects for other heavy atoms (^{13}C, ^{15}N, ^{17}O, etc.) can also be studied by natural abundance NMR using the same method. Unfortunately, there are still a number of unresolved problems in the natural abundance quantitative NMR analysis of these isotopes.[15] A further difficulty is that, since the isotope effects are much smaller than with deuterium, the analyses would need a much higher precision than for deuterium. For instance, while the range of variation of $^{13}C/^{12}C$ isotopic ratios at natural abundance is only of the order of 40‰ as compared with more than 500‰ for ^{2}H, for the precise determination of the specific ^{13}C contents of different sites in a molecule, the error should be better than 0.5‰. This is an extremely difficult task for ^{13}C NMR. Many factors can influence quantitative ^{13}C NMR analysis. The results of intramolecular ^{13}C ratio measurement may vary according to decoupling parameters such as the decoupling frequency offset (O2). In addition, in precise quantitative ^{13}C NMR analysis at natural abundance, the ~1.1% bi-labelled molecular species should be taken into account. Only recently have satisfactory quantitative ^{13}C NMR results been reported.[16] The approach described relied on the use of references of known site-specific isotopic ratio to determine the appropriate acquisition parameters. However, this approach may be suitable only in a limited number of cases as the same parameters cannot be applied to the analysis of all molecules. The quantitative analysis of ^{15}N, ^{17}O, etc., is even more of a challenge. In spite of the considerable progress made, NMR still cannot be used as a routine analytical tool for the precise determination of heavy atom isotope (^{13}C, ^{17}O, etc.) KIEs. However, in certain cases the KIE of carbon, oxygen or nitrogen can be determined at natural abundance with high precision using isotope ratio mass spectrometry, for isotopic analysis. This work is in progress.

EXPERIMENTAL

Bromination of alkylbenzenes. *Bromination of toluene with bromine.* A 1.7 ml (0.033 mol) amount of bromine was added slowly to 250 ml (2.35 mol) of toluene at 50 °C. The hydrogen bromide formed was conveyed to two successively connected traps containing pyridine by the nitrogen gas bubbling through the reaction mixture. The pyridinium bromide was isolated by evaporation of the pyridine and was dried under vacuum for 6 h. The excess toluene was removed by distillation under vacuum and the benzyl bromide was isolated by vacuum distillation.

Bromination of toluene with N-bromosuccinimide. A 6 g (0.033 mol) amount of *N*-bromosuccinimide was added to 250 ml (2.35 mol) of toluene under reflux (110 °C). Complete reaction was indicated by a negative starch–iodide test. The reaction flask was cooled to 0 °C and the succinimide was removed by filtration and dried under vacuum for 6 h. The excess toluene was removed by distillation under vacuum and the benzyl bromide was obtained by vacuum distillation.

Friedel–Crafts reactions. A 250 ml (2.8 mol) amount of benzene and 6.4 g (0.048 mol) of finely powdered anhydrous aluminium chloride were heated to 50 °C then

2.8 ml (0.04 mol) of acetyl chloride were added over a period of 30 min. The hydrogen chloride formed was conveyed to the traps by nitrogen gas bubbling as described above. After cooling, the pyridinium chloride was isolated by evaporation from the pyridine and was dried under vacuum for 6 h. After work-up, the excess benzene was removed by distillation and the acetophenone was purified by vacuum distillation.

Bromination of olefins. A 1.5 ml (0.028 mol) amount of bromine dissolved in 25 ml of carbon tetrachloride was added slowly to 200 ml (1.97 mol) amount of cyclohexene at 20 °C. The excess cyclohexene was removed by distillation under vacuum and (*E*) 1,2-dibromocyclohexane was isolated after vacuum distillation.

Diels–Alder reactions. A mixture of 3.7 ml (0.023 mol) of hexachlorocyclopentadiene and 200 ml (1.6 mol) of hex-1-ene was refluxed (65 °C) over a period of 20 days. The cycloaddition product was recovered by distillation under vacuum from the excess hex-1-ene.

Esterifications. While gently refluxing at constant temperature (EtOH, 78 °C; PrOH, 97 °C), 5.5 ml (0.047 mol) of benzoyl chloride were added dropwise to an excess of anhydrous alcohol (EtOH, 200 ml, 3.4 mol; PrOH, 250 ml, 3.34 mol). The refluxing was continued for 45 min to ensure complete reaction. After cooling, the excess alcohol was removed by distillation. The benzoate ester was isolated after evaporation under vacuum from the remaining alcohol.

NMR analysis. *NMR conditions.* The deuterium NMR spectra were recorded at 61.4 MHz under broad-band proton decoupling using a Bruker DPX 400 spectrometer equipped with an ^{19}F lock device; frequency window 1200 Hz, memory size 16K, scan number 200 to 27 000 according to the sample and exponential multiplication corresponding to a line broadening of 0.5 or 1 Hz. Six spectra were recorded for each sample and an average $(D/H)_i$ value was calculated from the six measurements.

$(D/H)_i$ calculation. The $(D/H)_i$ values were determined using an external reference, TMU (tetramethylurea)[†], of which the isotopic ratio $(D/H)_R$ was precisely calibrated. In the analysis of succinimide, dioxane was used as reference in place of TMU. $(D/H)_i$ was calculated from the following equation:

$$(D/H)_i = (D/H)_R P_R m_R M_S A_i / (P_i m_S M_R A_R) \qquad (15)$$

where P_i and P_R are the stoichiometric numbers of hydrogens in site *i* of the sample and in the reference and M_S, m_S, M_R and m_R are, respectively, the molecular weight and mass of the samples and of the reference. When detectable impurities are present, m_S (sample weight) in Eqn. (15) is replaced by $f_S m_S$, where f_S is the sample purity in mole fraction. A_i and A_R are, respectively, the signal areas of the site *i* monodeuterated molecule and the reference. These were derived from the quantitative evaluation of the ^2H NMR spectrum using a curve-fitting program (Interliss from Eurofins Scientific, Nantes, France).[13]

Sample preparation. The purity of all analysed samples was checked by GC. If the NMR signals of the impurity did not disturb the sample' spectrum, no further purification was carried out. The liquid samples were mixed directly with the reference and lock materials without solvent. NMR tubes of 10 mm diameter were used. For the determination of $(D/H)_{NH(HBr\ or\ HCl)}$ values, the solid samples (succinimide and pyridinium halides) were dissolved in a mixture of deuterium-depleted water [(D/H) = 3 ppm] and acetonitrile (1:2.5) and deuterium-depleted water and pyridine (1:3), respectively. In the calculation of $(D/H)_{NH}$ values, a correction was made for the contribution of residual deuterium in the deuterium depleted water.

[†] The reference material TMUC (STA003) can be purchased from IRMM, Retieseweg 13-2440 Geel, Belgium.

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