# Evidence for an NIH Shift as the Origin of the Apparently Anomalous Distribution of Deuterium in Estragole from *Artemisia dracunculus*

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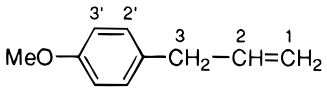
*Received September 17, 1999*

Feeding experiments of [2-$^2$H]- and [4′-$^2$H]phenylalanine in *Artemisia dracunculus* validate the hypothesis that the marked difference in deuterium content at the natural abundance level between the aromatic carbons of estragole (**1**) is due to an NIH shift during the hydroxylation of the benzene ring.

It is well recognized that the relative natural abundance of deuterium at distinct sites within any metabolite results from kinetic and thermodynamic isotope effects involved in the formation of the final product.[1,2] The site-specific natural isotope fractionation (SNIF) of hydrogen reckoned by nuclear magnetic resonance[3] has been exploited to identify the synthetic origin or the biological source of a given product.[4−7] In addition, the SNIF-NMR method is a very attractive tool to provide information on the biosynthesis of natural products, in particular on inter- and intramolecular hydrogen transfers.[8] However, any interpretation of apparent anomalies in the relative abundance of deuterium requires the support of careful tracer experiments.[9]

In this paper we report experimental evidence in favor of an NIH shift[10−12] as the cause of the relatively high deuterium content at the carbon atoms adjacent to the methoxy group in the biosynthesized estragole molecule (**1**).



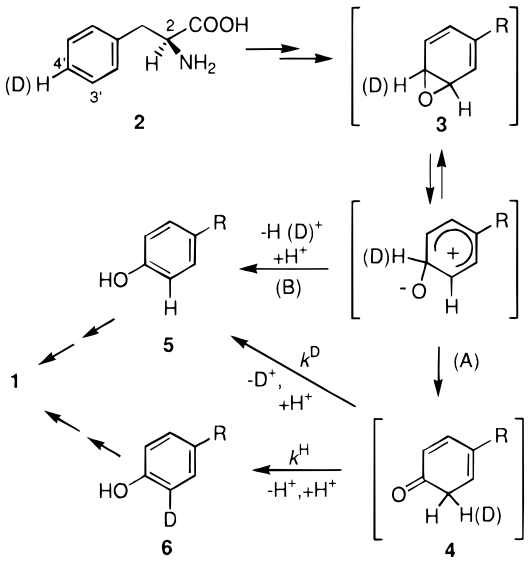

**1**

Estragole (=methylchavicol) (**1**) is the main constituent of the essential oil of *Artemisia dracunculus* L. (Asteraceae) (tarragon),[13,14] which represents a natural source of this compound used in the flavor and fragrance industry.[13]

The $^2$H NMR spectrum of estragole (**1**) from the essential oil of *A. dracunculus* cultivated in Piedmont (Italy) showed marked differences in deuterium abundance between the individual positions of the $C_6-C_3$ skeleton (see Table 1, expt 1). In particular, on the aromatic ring, the C-3′,5′ site appears to be richer in deuterium than the C-2′,6′ site; in fact, the distribution factor $R^1$ with respect to the methyl group is 2.94 for the former and 1.86 for the latter (the statistical values being 2 for both sites and 3 for the methoxy group). Similar *R* values have been reported for estragole extracted from fennel, chinese anise-tree, and turpentine.[15,16]

Such a considerable deuterium enrichment could be explained in terms of a partial migration of hydrogen from the C-4′ position of allylphenol precursors to the adjacent one during the in vivo hydroxylation of the aromatic ring.[10−12] The spontaneous isomerization of an arene oxide (e.g., **3**) followed by a non-stereoselective tautomerization

**Scheme 1.** Hydroxylation of the Aromatic Ring and Associated NIH Shift during the in Vivo Conversion of l-Phenylalanine (**2**) into Estragole (**1**) via *p*-Coumaric Acid (**5**, **6**)




R = −CH=CH−COOH (*E*)

of the resulting ketodiene (e.g., **4**) is generally recognized as the origin of the migration to and retention of a hydrogen isotope at the neighboring position relative to the labeled position in the starting arene substrate (Scheme 1, path A). This phenomenon is known as the "NIH shift"[10] and has been observed during a great number of enzyme-catalyzed hydroxylations in microorganisms,[12] plants,[17−20] and animals.[20] Considering the presence of only monodeuterated molecules ($^2$H/$^1$H being ca. $1.5 \times 10^{-4}$ in natural compounds)[21] at all levels along the pathway leading from l-phenylalanine (**2**) to estragole (**1**)[22] and a strong prevalence of path A showing primary kinetic isotope effect ($k^H/k^D > 1$) for the enolization step (Scheme 1), the $^2$H(3′,5′)/$^2$H(2′,6′) ratio for each 4′-hydroxylated intermediate, most probably coumaric acid (**5**, **6**) and sequential metabolites, is expected to be significantly higher than that for non-hydroxylated precursors.

The following experiments were carried out to confirm the involvement of an NIH shift in the biosynthesis of estragole (**1**). Two preliminary tests served to set feeding conditions for satisfactory incorporations of deuterium-substituted D,L-phenylalanines into estragole in *A. dracunculus*. No significant change in deuterium distribution

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10.1021/np990457q CCC: $19.00 
Published on Web 04/13/2000

**Table 1.** Deuterium Distribution in Estragole (**1**) Resulting from Precursor-Incorporation Experiments on *A. Dracunculus* L.

| | | | | | | | relative abundance of monodeuterated molecules[c] $^2$H-containing sites | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| expt | compound fed (A) | amount of A (g) | total weight (g) of plants | estragole (g)[a] | incorporation (%)[b] | OMe | C-3′,5′ | C-2′,6′ | C-3 | C-2 | C-1 |
| 1 | none | | 290 | 0.36 | | 1 | 0.98 | 0.62 | 0.83 | 0.37 | 0.77 |
| 2 | D,L-Phe | 1.00 | 480 | 0.58 | | 1 | 0.99 | 0.61 | 0.83 | 0.38 | 0.75 |
| 3 | D,L-[2-$^2$H]Phe (98 at. % $^2$H) | 1.00 | 430 | 0.67 | 0.12 | 1 | 0.96 | 0.59 | 0.80 | 2.15 | 0.75 |
| 4 | D,L-[2,4′-$^2$H]Phe $^2$H(4′)/$^2$H(2)=1.35[d] | 1.15 | 850 | 0.83 | 0.21 | 1 | 2.18 | 0.61 | 0.84 | 1.61 | 0.73 |

[a] Estimated by quantitative GC analysis of the essential oil. [b] Calculated on L-[2-$^2$H]Phenylalanine. [c] Relative peak intensities in $^2$H NMR spectra. Five spectra were recorded for each sample; the average values for the single intensity ratios were characterized by SDs in the range 0.03−0.08. [d] Calculated from $^2$H NMR of the feeding mixture containing 98 and 95 atom % $^2$H in [2-$^2$H] and in [4′-$^2$H]Phe, respectively.

was observed when a substantial amount of D,L-phenylalanine was absorbed by tarragon shoots (compare data from expts 2 and 1 in Table 1). Under the same conditions incorporation of D,L-[2-$^2$H]phenylalanine was found to be modest (expt 3) but enough for $^2$H NMR analyses. Note that on the basis of the quantity of estragole produced (taken as entirely derived from endogenous L-phenylalanine), assuming the average deuterium content on each C−H bond (e.g., in the reference Me group) to be $1.46 \times 10^{-4}$,[16] and taking into account the complete retention of deuterium in the transformation of L-[2-$^2$H]phenylalanine,[23] the precursor incorporation could approximately be calculated from $^2$H NMR data (see Table 1).

When a mixture of D,L-phenylalanines substituted with deuterium at the 2- or 4′-position was administered to *A. dracunculus* (expt 4), the isolated estragole (**1**) showed a $^2$H NMR spectrum with peak intensities as reported in Table 1. On the basis of the deuterium distribution values for estragole observed in expts 2 and 4, the ratio between the excesses of molecules monodeuterated at the 3′,5′- and 2-position obtained from expt 4 was found to be $0.97 \pm 0.08$. Since the theoretical value was $1.35 \pm 0.05$, assuming the same percentage of incorporation for both the substrates supplied in the mixture and a complete deuterium retention, the actual percentage of deuterium migration from the 4′-position in L-phenylalanine to the 3′,5′-position in the allylphenol (**1**) could be estimated as $71.6 \pm 6.4$. This value is in agreement with values for tritium retention observed in a number of in vivo incorporations of L-[4′-$^3$H]-phenylalanine into (4′-hydroxy)phenylpropanoids (80−90%),[10,17−20] provided the relation between tritium and deuterium isotope effects is considered.[10,24]

In light of the above results, the marked deuterium enrichment observed in the 3′,5′-position of natural estragole (**1**) can be explained by an NIH shift following a mono-oxygenase-catalyzed hydroxylation of the benzene ring. It should be noted that in some procaryotes a dioxygenase-catalyzed hydroxylation of the arene nucleus has been found to involve the NIH shift through an alternative mechanism, i.e. via dehydration of *cis*-dihydrodiol intermediates to ketodienes without involvement of arene oxides.[12]

## Experimental Section

**General Experimental Procedures.** $^1$H (300.13 MHz) and $^2$H (46.07 MHz) NMR spectra were recorded on a Bruker AC 300 spectrometer in $CCl_4$, with TMS as internal reference. The $^2$H NMR spectra were obtained in the $^1$H decoupled mode (broad band) with a 5.8 s pulse repetition time and a 90° flip angle at 304 K. Good signal-to-noise ratios were obtained for concentrated solutions (estragole−$CCl_4$, 3:1 v/v) using a 10 nm OD sample tube. The sweep width was 100 Hz. EIMS spectra were obtained on a VG 7070 EQ mass spectrometer operating at 70 eV. GC analyses were carried out on a DANI 3880 gas chromatograph equipped with a FID using a homemade glass column (2m × 2 mm i.d.) packed with 5% LAC 767 on Chromosorb W (60−80 mesh). The GC parameters were as follows: carrier gas, $N_2$ at 30 mL/min; injector temperature, 210 °C; detector temperature, 220 °C; the oven temperature was held at 100 °C for 8 min, then increased to 200 °C at 10 °C/min, and held at 200 °C for 8 min. TLC was performed on silica gel $F_{254}$ precoated aluminum sheets (0.2 mm layer, Merck). Silica gel (63−200 $\mu$m) from Merck was used for column chromatography. D,L-[2-$^2$H]Phenylalanine (98 atom % $^2$H) was from MSD Isotopes (München, Germany); deuterium (99.8 atom % $^2$H) and D,L-(4-bromophenyl)alanine were from Aldrich.

**Plant Material.** Plants of *A. dracunculus* L. were cultivated in the field for 3 months (ca. 20 cm in height) and harvested in May. A voucher specimen is deposited at the Department of Organic and Industrial Chemistry, University of Milan, Italy.

**Extraction and Isolation Procedure.** The plant material was cut into small pieces and steam distilled. Extraction of the distillate (500 mL) with ethyl ether and evaporation of the solvent gave an oil (typically 1 mL per 500 g fresh material) containing 60−70% estragole (GC analysis using anethole as internal standard, $t_R$ estragole 11.3 min; $t_R$ anethole 14.2 min). The essential oil was then chromatographed on a silica gel column. Fractions eluted with petroleum ether (bp 40−70 °C)−toluene (2:3) afforded estragole, which was checked for purity by TLC (eluent $CHCl_3$) and GC; $^1$H NMR (300 MHz, $CCl_4$) $\delta$ 3.21 (1H, d, $J$ = 6.7 Hz, $H_2$-3), 3.55 (3H, s, OMe), 4.94−5.00 (2H, m, $H_2$-1), 5.79−5.90 (1H, m, H-2), 6.69 (2H, d, $J$ = 8.5 Hz, H-3′, H-5′), 6.95 (2H, d, $J$ = 8.5 Hz, H-2′, H-6′).

**Administration of D,L-Phenylalanine to Shoots of *A. dracunculus*.** Phenylalanine (ca. 1 g) was dissolved in distilled water (100−200 mL) and the pH adjusted to 8.5 by addition of $NaHCO_3$. The clear solution was divided equally among 20−40 test tubes. Vigorous shoots of *A. dracunculus* (15−25 cm long) were cut under water, and the cut ends were immediately immersed into the solutions in the feeding tubes. When the shoots had absorbed almost all the solution, the remainder was continuously "washed in" with distilled water until the shoots were harvested 3 h later. Batches of cuttings treated equally were worked up as described in the previous section.

**Preparation of D,L-[4′-$^2$H]Phenylalanine.** The title compound was prepared in 70% yield by hydrogenolysis of D,L-(4-bromophenyl)alanine with $D_2$ using Pd/$CaCO_3$ (5%).[25] The chemical purity of the recrystallized product was checked by TLC (eluent 2-PrOH−HOAc−$H_2O$, 4:1:1); [$^2$H]-species $95 \pm 1$% by MS; $^1$H NMR (300 MHz, $CCl_4$) $\delta$ 2.66 (1H, dd, $J$ = 7.3, 13.4 Hz, H-3a), 2.81 (1H, dd, $J$ = 5.7, 13.4 Hz, H-3b), 3.32 (1H, dd, $J$ = 5.7, 7.3 Hz, H-2), 7.10 (2H, d, $J$ = 8.0 Hz) and 7.21 (2H, d, $J$ = 8.0 Hz) (aromatic-H).

A sample of the above D,L-[4′-$^2$H]phenylalanine (0.67 g) was added to commercial d,L-[2-$^2$H]phenylalanine (0.48 g), and the mixture dissolved in water. A small portion of this solution was analyzed by $^2$H NMR to determine the $^2$H(4′)/$^2$H(2′) ratio.

The remaining solution was used to carry out the feeding experiment (expt 4 of Table 1).

**Acknowledgment.** Thanks are due to MURST (Progetto: Chimica dei Composti Organici di Interesse Biologico) for financial support and Dr. Franco Chialva for supplying plant material.

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NP990457Q