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# In vivo transformations of dihydro-*epi*-deoxyarteannuin B in *Artemisia annua* plants

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**Abstract**—[15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**) has been fed to intact *Artemisia annua* plants via the root and three labeled metabolites (**17a**–**19a**) have been identified by 1D- and 2D-NMR spectroscopies. The in vivo transformations of **4a** in *A. annua* are proposed to involve enzymatically-mediated processes in addition to possible spontaneous autoxidation. In the hypothetical spontaneous autoxidation pathway, the tri-substituted double bond in **4a** appears to have undergone 'ene-type' reaction with oxygen to form an allylic hydroperoxide, which subsequently rearranges to the allylic hydroxyl group in the metabolite 3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (**17a**). In the enzymatically-mediated pathways, compound **17a** has then been converted to its acetyl derivative, 3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (**18a**), while oxidation of **4a** at the 'unactivated' 9-position has yielded 9β-hydroxy-dihydro-*epi*-deoxyarteannuin B (**19a**). Although all of the natural products artemisinin (**1**), arteannuin K (**7**), arteannuin L (**8**), and arteannuin M (**9**) have been suggested previously as hypothetical metabolites from dihydro-*epi*-deoxyarteannuin B in *A. annua*, none were isolated in labeled form in this study. It is argued that the nature of the transformations undergone by compound **4a** are more consistent with a degradative metabolism, designed to eliminate this compound from the plant, rather than with a role as a late precursor in the biosynthesis of artemisinin or other natural products from *A. annua*.


## 1. Introduction

The Chinese plant *Artemisia annua* L. (sweet wormwood; Compositae) is the source of the potent antimalarial sesquiterpene, artemisinin (qinghaosu) (**1**).[1] Several attempts have been made to define the biogenesis of the 1,2,4-trioxane ring which is responsible for the antimalarial activity of **1**, and most investigators have concurred that either dihydroartemisinic acid (**2**)[2–9] (Fig. 1) or its 11,13-dehydro analogue, artemisinic acid (arteannuic acid),[10] is the late-stage precursor en route to artemisinin. (A biosynthetic study with artemisinic acid is described in the companion paper to this manuscript.)[11]

In a recent detailed study of the biogenesis of artemisinin,[8] employing a stable isotope-labeled precursor which was fed via the root to intact plants of *A. annua*, and using $^{2}H$ NMR spectroscopy to directly analyze the products of metabolism, it was shown that dihydroartemisinic acid (**2**) is first converted to the tertiary allylic hydroperoxide **3** in vivo (Fig. 1). Compound **3** was then proposed to be the key intermediate[†] in the biogenesis of artemisinin (**1**) and a further 15 labeled metabolites that were isolated from *A. annua*. The three most abundant metabolites recovered from the further in vivo transformations of **3** were dihydro-*epi*-deoxyarteannuin B (**4**),[3,8,12,13] dihydroarteanuin B (**5**),[3,8,13,14] and the *seco*-cadinane sesquiterpene **6**[8,13,15] (in vivo transformations leading to and from **3** which are shown by bold arrows in Fig. 1 have been confirmed experimentally). Given the relative abundance of the three metabolites **4**, **5**, and **6** compared to artemisinin (**1**), it was considered possible that any one of these compounds might itself be a later stage biosynthetic intermediate en route from **3** to artemisinin (**1**), as shown by the dashed arrows in Figure 1. Indeed, experimental evidence for the top dashed arrow in Figure 1, from dihydro-*epi*-deoxyarteannuin B (**4**) to artemisinin (**1**) (labeled 'Refs. 8 and 16'), has been provided from previous studies, involving cell-free extracts and

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[†] In this in vivo experiment,[8] the concentration of compound **3** initially increased in the extracts of *A. annua* at a rate which closely matched the rate of disappearance of dihydroartemisinic acid (**2**); the concentration of **3** then peaked and subsequently declined at a rate which closely mirrored the appearance of the sum of all the other metabolites (compounds **4**, **5**, and **6** being the most abundant). Such behavior can most easily be explained if compound **2** is being transformed into **4**, **5**, **6**, and the other metabolites via **3** as an intermediate.

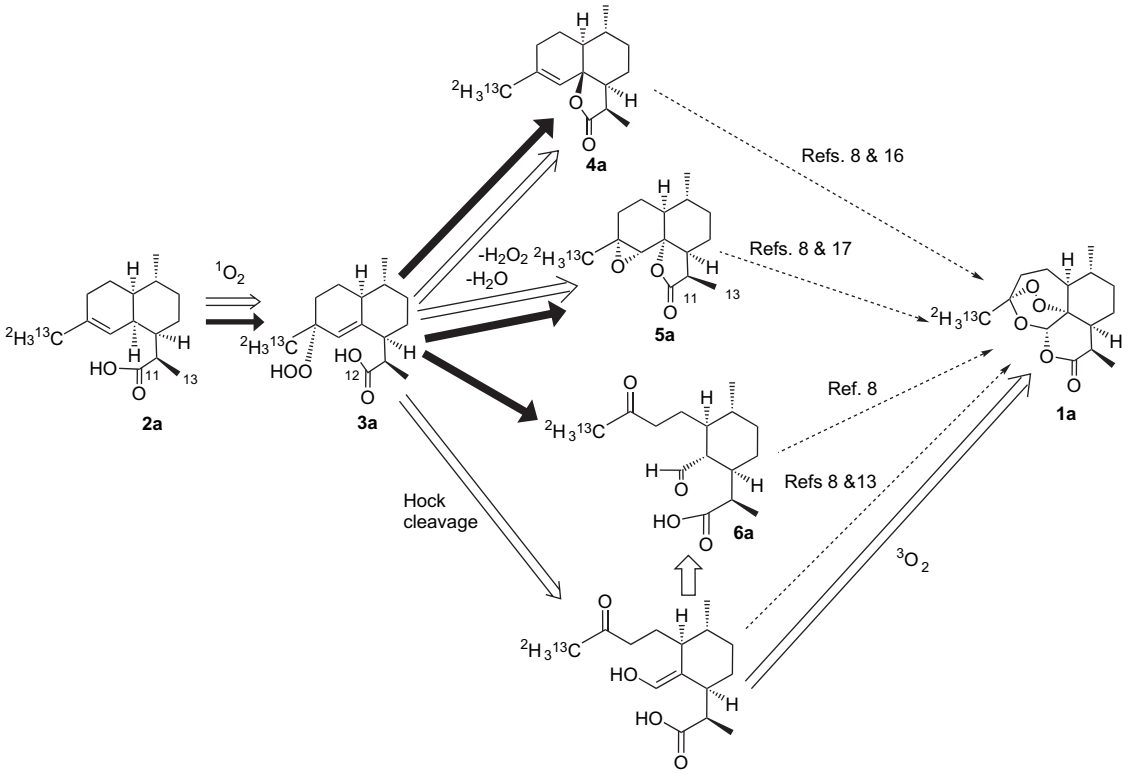



**Figure 1**. Conversion of dihydroartemisinic acid (**2**) to artemisinin (**1**) via the intermediate tertiary allylic hydroperoxide **3**. Bold arrows represent transformations of [15-$^{13}C^2H_3$]-dihydroartemisinic acid (**2a**), which have been shown to occur in vivo in *Artemisia annua*.[8] Open arrows represent processes which have also been shown to occur spontaneously in vitro.[13] Dashed arrows indicate hypothetical biogenetic routes to **1** which have been proposed to occur in vivo by enzymatic transformations of the late-stage precursors **4**,[15] **5**[16] or **6** (or its enol tautomer).[8] The suffix 'a' indicates that the [15-$CH_3$] group of a metabolite has been replaced by [15-$^{13}C^2H_3$].

radioisotopically-labeled precursors, which were performed by Wang et al.[16] The literature also contains experimental evidence from Jain et al.[17] that enzyme systems from *A. annua* can convert dihydroarteannuin B (**5**) into artemisinin (**1**), as shown by the next dashed arrow in Figure 1 (labeled 'Refs. 8 and 17'). Even more confusingly, it has, in addition, been proposed by Nair and Basile[18] that the 11,13-dehydro analogue of dihydroarteannuin B (**5**), arteannuin B, can be converted into artemisinin by *A. annua*—although Wang et al.[19] were unable to confirm this (this point is discussed further in the companion paper).[11]

There was reason to believe that all three compounds **4**, **5**, and **6**—the most dominant metabolites from dihydroartemisinic acid (**2**) in vivo—might have been formed by reactions which are expected to occur spontaneously for hydroperoxides such as **3**.[1,20] Indeed, the formation of each of the metabolites **4**, **5**, and **6** had been explicitly demonstrated in vitro in a previous study of the spontaneous autoxidation of dihydroartemisinic acid (**2**).[13] In this in vitro study, compound **2** was initially transformed into the intermediate **3** (as indicated by the open arrows in Fig. 1), in just the same manner as has been observed in vivo.[8] Subsequent intramolecular $S_N2'$ attack of the 12-$CO_2H$ group at the allylic hydroperoxide group in **3** gave rise to **4**, while an alternative rearrangement of the allylic hydroperoxide to a β-epoxy alcohol, followed by lactonization, gave rise to **5**, and a third alternative Hock cleavage reaction of the allylic hydroperoxide **3** produced the enol shown at the bottom of Figure 1, which was actually isolated as its aldehyde tautomer **6**. (Experimental evidence was also obtained that this unstable enol could be converted directly to artemisinin (**1**) in vitro in the presence of atmospheric oxygen.)[13]

Small amounts of labeled arteannuin K (**7**),[3,15] arteannuin L (**8**),[3,15] and arteannuin M (**9**)[3,15] (Fig. 2) were found amongst the 15 metabolites which had been recovered from the feeding of dihydroartemisinic acid (**2**) to intact *A. annua* plants.[8] It has previously been hypothesized that autoxidation reactions, occurring at the tri-substituted double bond in dihydro-*epi*-deoxyarteannuin B (**4**), which were similar in nature to those observed for **2**, might account for the biogenesis of arteannuins K (**7**), L (**8**), and M (**9**)[3,15,21] in vivo. Once again, there was some evidence from in vitro studies[21] to support such a pathway, which would involve the intermediacy of secondary allylic hydroperoxides, formed during the spontaneous autoxidation of **4** (Fig. 2); indeed, one of the three possible natural products, arteannuin K (**7**), had actually been isolated from further hypothetical transformations of such a hydroperoxide in vitro. However, because of the relatively small amounts of metabolites **7**–**9** which were obtained from the in vivo feeding study with dihydroartemisinic acid (**2**),[8] there was insufficient evidence to confirm whether any of the three metabolites **7**–**9** had actually been derived directly from **4** by this kind of spontaneous autoxidation reaction, occurring in the tissues of *A. annua*. The proposal for the biogeneses of the natural products **7**, **8**, and **9** from **4**, which is shown in Figure 2, should therefore be regarded as more tentative than that for the formation of metabolites **4**, **5**, and **6** from **2**, which is shown in Figure 1.

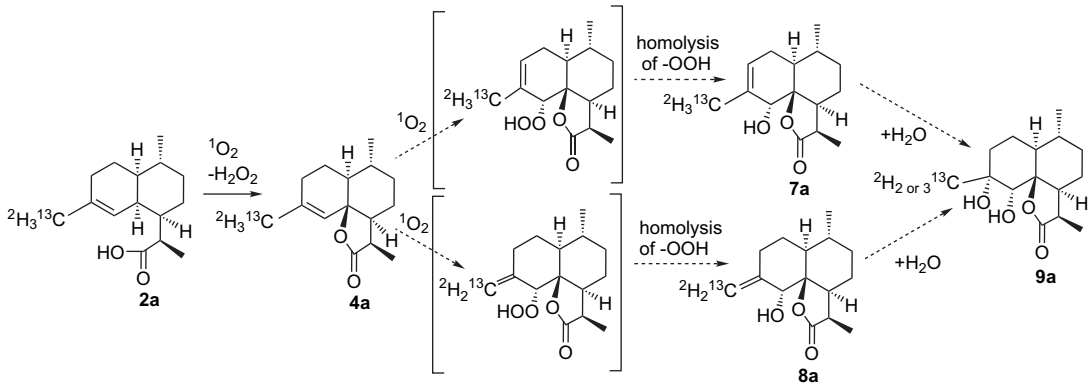



**Figure 2**. Tentative proposals which were made in Ref. 8 for the further in vivo transformations of dihydro-*epi*-deoxyarteannuin B (**4**) by spontaneous autoxidation processes similar to those shown in Figure 1. These proposals were based on the isolation of all of the isotopically-labeled compounds [15-$^{13}C^{2}H_3$]-arteannuin K (**7a**), [15-$^{13}C^{2}H_3$]-arteannuin L (**8a**), and [15-$^{13}C^{2}H_3$]-arteannuin M (**9a**) from *Artemisia annua* plants which had been fed with labeled [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**2a**). The suffix 'a' indicates that the [15-$CH_3$] group has been replaced by [15-$^{13}C^{2}H_3$].

## 2. Results and discussion

Having recently described a synthesis of doubly-labeled[21] [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**),[‡] it was resolved to perform a detailed feeding study, which was designed to answer the two questions raised in the foregoing discussion, regarding the biogenetic status of compound **4** in *A. annua*. Firstly, is dihydro-*epi*-deoxyarteannuin B (**4**) a late-stage intermediate en route from **3** to artemisinin (**1**) (upper dashed arrow labeled 'Refs. 8 and 16' in Fig. 1)? As discussed above, this proposal is not inconsistent with this detailed in vivo study of the biostransformations undergone by dihydroartemisinic acid (**2**) in *A. annua*,[8] and it is supported by the earlier results of Wang et al.[16] Or, alternatively, is dihydro-*epi*-deoxyarteannuin B the in vivo precursor to some or all of arteannuins K (**7**), L (**8**), and M (**9**) (Fig. 2)? Such a biosynthetic pathway has been suggested by previous in vitro studies with compound **4**[21] and—once again—would not be inconsistent with the recent in vivo study, in which dihydroartemisinic acid (**2**) was fed to *A. annua*.[8]

The choice of a stable isotope-labeled precursor for these feeding experiments—[15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B, containing both $^{13}C$ and $^{2}H$ labels[21]—has several advantages over the radioisotopically-labeled precursors which have been employed almost exclusively in earlier studies of the metabolism of *A. annua*.[10,16–19] Firstly, one-dimensional (1D-) $^{2}H$ NMR studies (Section 2.4) can give preliminary information directly from a crude plant extract (via the $^{2}H$ chemical shift) about the nature of the transformations of **4a** which have occurred in *A. annua*, without the necessity for prior chromatographic separation. Secondly, by use of the novel two-dimensional (2D-) $^{13}C$–$^{2}H$ correlation NMR spectroscopy experiment ($^{13}C$–$^{2}H$ COSY),[8,22,23] these same labeled metabolites can also be identified in the crude extracts from their $^{13}C$ chemical shifts. (This technique relies on the sizeable one-bond coupling constant which is expected between the $^{13}C$ resonance and the $^{2}H$ resonance at the 15-position.) The availability of two NMR parameters ($\delta_C$ and $\delta_D$) then provides a greater degree of confidence when attempting an identification of an individual metabolite in the crude plant extract under analysis (Section 2.4).[8,22] Finally, the presence of the $^{2}H$ label greatly facilitates the chromatographic separation, which is required to obtain pure labeled metabolites for a full chemical characterization (Section 2.5).[8] Because of the very low natural abundance of $^{2}H$ (0.015%), only those chromatographic fractions which contain metabolites that have been generated from the biotransformations of **4a** will give a signal in $^{2}H$ NMR spectroscopy; hence, it is possible to make a very rapid screening of chromatographic fractions by NMR, selecting only those which show a characteristic $^{2}H$ doublet signal as candidates for further purification.

When performing metabolic studies with **4a**, it was decided to feed this labeled precursor to intact *A. annua* plants via the root, as for the previous study of the metabolism of labeled dihydroartemisinic acid (**2a**) in *A. annua*.[8] This is, in the opinion of the author, one of the most 'natural' scenarios for studying biotransformations of any labeled precursor, such as **4a**, in vivo. Such a feeding technique should certainly be less prone to the creation of artifacts than the use of cell-free extracts, which have commonly been employed by most other researchers when investigating the biogenesis of artemisinin. As discussed in Section 2.1, compound **4** is susceptible to spontaneous oxidation (see also Fig. 3).[21,22,24] Hence, the adoption of the root-feeding technique was considered particularly critical in order to minimize the possibility for introducing artifacts from the autoxidation of **4**, which are considered much more likely to occur through direct exposure to oxygen in the atmosphere during the extensive manipulations of the biological source material that are associated with the preparation of cell-free extracts and their use as an assay system.

### 2.1. Preparation of isotopically-labeled dihydro-*epi*-deoxyarteannuin B (4a)

Previous experiences when performing biosynthetic experiments with intact *A. annua* plants,[8,22,23] have indicated that the stable isotope-labeled precursor must be of both high chemical purity and high isotopic enrichment when feeding to living plants. Fortunately, a synthesis of dihydro-*epi*-deoxyarteannuin B which has been described recently[21]

[‡] Throughout this paper, the suffix 'a' indicates the presence of a [$^{13}C^{2}H_3$] label at the 15-position.

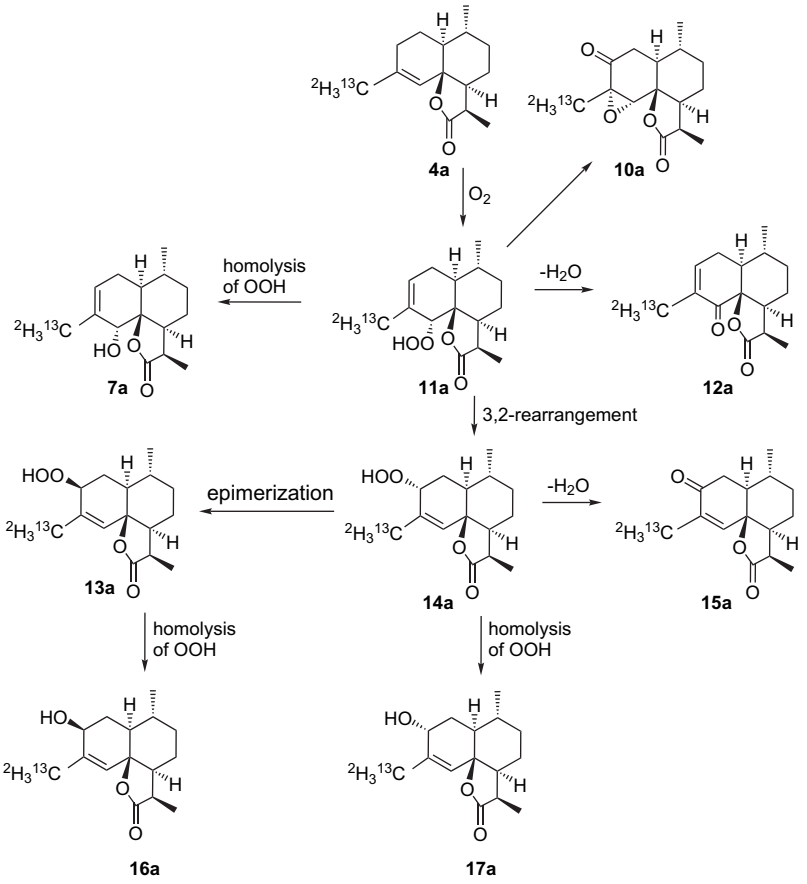



**Figure 3**. Products isolated from the re-purification by HPLC of compound **4a**, prior to the commencement of feeding experiments with *Artemisia annua* plants. All of the products **7a** and **10a**–**17a** are proposed to be formed by the spontaneous autoxidation of **4a** which occurs upon prolonged storage.

permitted the preparation of such a sample of [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**) in good yield and with almost 100% enrichment of both the $^{2}H$ and $^{13}C$ labels at the 15-position (as determined both by $^{13}C$ and $^{2}H$ NMR spectroscopies and by mass spectrometry). As noted previously,[21] compound **4a** is susceptible to spontaneous autoxidation when subjected to prolonged storage and it was suggested that this compound should therefore be re-purified just prior to its use in any feeding experiment.[§] As expected, HPLC separation of a sample of **4a** which had been subjected to storage yielded all of the autoxidation products that have been described before (compounds **7a** and **10a**–**15a**),[21] as well as very small amounts of two allylic hydroxides (compounds **16a** and **17a**) which had previously escaped detection. Both compounds **16** and **17** are proposed to be derived from the spontaneous autoxidation reaction of **4** to the secondary allylic hydroperoxide **11**,[21] followed by a 3,2-rearrangement and homolysis of the resultant hydroperoxides, as is shown in Figure 3.

## 2.2. Determination of the optimum method for feeding [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (4a) to intact *A. annua* plants

Dihydro-*epi*-deoxyarteannuin B (**4**) is a hydrophobic compound which is essentially insoluble in water, and it was therefore necessary to include an emulsifying agent in order to enhance the solubility of this labeled precursor in the aqueous feeding medium.[¶] The non-ionic detergent, Triton®, was found to be an effective solubilizing agent for **4a**, which gave good results in feeding experiments when employed at comparatively low concentrations. Since Triton® is known to be able to disrupt cell membranes, care was taken to use the minimum amount of this detergent which was consistent with obtaining a reasonably concentrated feeding solution of compound **4a** (ca. 1 mg/ml), although, in the event, it appeared that Triton® did not pass through the physiological barrier of the root cortex (Section 4.3.1).

## 2.3. Feeding and extraction of *A. annua* plants

Seven intact *A. annua* plants were fed individually via the root with an aqueous feeding solution of **4a**, which contained a minimal amount of Triton®. The plants were then kept alive under hydroponic conditions and each individual plant was harvested at intervals of a few days, separated into an aerial compartment (consisting of leaf and stem) and a root compartment, then extracted by organic solvent ($CH_2Cl_2$ followed by MeOH). The experiment was completed after

[§] This was considered to be particularly important as arteannuin K (**7**),[15] one of the products isolated from the spontaneous autoxidation of **4**,[21] has previously been reported as a natural product from *A. annua*.[3]

[¶] There is no organic acid functional group present in compound **4** which can be used to increase its solubility at alkaline pH, as was the case for the previous experiments when feeding dihydroartemisinic acid (**2**)[8] or artemisinic acid,[11] to intact *A. annua* plants via the root or cut stem.

two weeks when the last plant had been harvested and extracted.

There was no discernible trend in the amount of $^{2}H$ label which was recovered from the crude extracts of the aerial compartment of *A. annua* over the course of the experiment (the recovery varied from 10 to 50% of the total amount of label which had been administered). In addition to this unexpected variability, the absolute amounts of **4a** and its metabolites which were recovered from the organic extracts were significantly lower than in our previous biosynthetic experiment, which had also employed root-feeding of the precursor dihydroartemisinic acid (**2a**) (dissolved in TRIS buffer) to intact *A. annua* plants.[8] In spite of this, the extent of the recovery of label from the feeding of **4a** in this experiment was still sufficiently high that labeled metabolites were easily detectable in all the crude extracts of the aerial parts, both by 1D-$^{2}H$ NMR and by 2D-$^{13}C$–$^{2}H$ COSY NMR spectroscopies, as is described in the next section.

Mass spectroscopic analysis of dihydro-*epi*-deoxyarteannuin B which was recovered from chromatographic purification of the combined aerial extracts from *A. annua* (see Section 2.5) revealed it to contain molecular ions for both the [15-$^{13}C^{2}H_3$]-labeled isotopomer (**4a**) ($M^+$ at *m/z* 238) and for the isotopically-normal form of **4** ($M^+$ at *m/z* 234), in a ratio of approximately 3:1. By contrast, the molecular ions for the partially labeled [15-$^{13}C^{2}H_2H$]- and [15-$^{13}C^{2}HH_2$]-isotopomers of **4** were present at much lower abundance. Since the synthetic precursor **4a** which had been administered to the plants was almost 100% deuteriated (Section 2.1), the low intensity of peaks due to the partially deuteriated isotopomers in the dihydro-*epi*-deoxyarteannuin B which was recovered from the feeding experiment, suggested that there had been little or no depletion of $^{2}H$ from the 15-position of **4a** as a consequence of metabolic processes occurring in the plant. It was concluded therefore that the isotopically-normal peak, which had been observed in the mass spectrum from the feeding experiment ($M^+$ at *m/z* 234) must represent the *bona fide* natural product, dihydro-*epi*-deoxyarteannuin B, which would have been present in *A. annua* in any case,[3,12,21] even if labeled **4a** had not been administered to the plants.

The amount of $^{2}H$ label extracted back from the root compartment was observed to decline from 12% of the total fed at the start of the experiment to 0% at the end. This may, perhaps, indicate that dihydro-*epi*-deoxyarteannuin B was being redistributed from the root to the rest of the plant over the course of the experiment, although there may also have been some unavoidable 'coating' of compound **4a** onto the surface of the root during the feeding period (i.e., not all of compound **4a** which was recovered from the root compartment may have been associated with actual uptake).

### 2.4. $^{2}H$ NMR and $^{13}C$–$^{2}H$ COSY NMR analyses of the crude $CH_2Cl_2$ extracts of *A. annua* plants which had been fed with [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (4a)

The successful uptake of the labeled precursor **4a** by each of the intact *A. annua* plants to which it had been fed was indicated by the appearance of a doublet[‖] resonance ($\delta_D$ 1.68 ppm, $^{1}J_{CD}$=19.1 Hz) in the 1D-$^{2}H$ NMR spectra of the crude extracts of the aerial compartment. (This signal was associated with the deuterium label at the 15-position of [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**).)[21] Plants extracted 68 h or more after feeding of **4a** also exhibited an additional doublet ($\delta_D$ 1.81 ppm, $^{1}J_{CD}$=19.5 Hz) in the $^{2}H$ NMR spectra recorded from their aerial parts. Although, at its first appearance, this new resonance was of quite low intensity (~10% as compared with the doublet due to compound **4a**), by the end of the experiment, this doublet accounted for roughly half of the label which was recovered from the aerial compartment (Fig. 4). No 'new' doublet peaks were observed to appear in the $^{2}H$ NMR spectra of any of the crude root extracts and it appears, therefore, that compound **4a** may not be metabolized in the roots.

At the end of the experiment, all the crude extracts of the aerial parts were combined together and analyzed by 2D-$^{13}C$–$^{2}H$ correlation NMR spectroscopy ($^{13}C$–$^{2}H$ COSY).[8,22,23] This novel analytical technique confirmed the presence of the metabolite which had been identified previously from its doublet signal at $\delta_D$ 1.81 ppm in 1D-$^{2}H$ NMR spectroscopy and, in addition, indicated that the $^{13}C$ chemical shift at the 15-position for this labeled metabolite was $\delta_C$ 19.9 ppm (Fig. 5).[**] A second metabolite of **4a** was also clearly identified by a weaker 2D-signal, appearing in the $^{13}C$–$^{2}H$ COSY spectrum at $\delta_D$ 1.69/$\delta_C$ 19.6 ppm. This metabolite had not been well resolved in the previous 1D-$^{2}H$ NMR analyses, because its deuterium signal overlapped with that of **4a** in the $^{2}H$ dimension. It was also apparent from Figure 5 that the 2D-peak for **4a**, which is the most dominant signal in the $^{13}C$–$^{2}H$ COSY spectrum, might contain a small downfield 'shoulder' in the $^{13}C$ dimension. Although this could perhaps be an artifact of either the acquisition or the processing of the $^{13}C$–$^{2}H$ COSY spectrum in the vicinity of such a relatively intense peak, it is also possible that this is a genuine feature of the spectrum, because a third minor metabolite was later isolated from the combined crude plant extracts (see Section 2.5), with the correct $^{2}H$ and $^{13}C$ chemical shifts at the 15-position ($\delta_D$ 1.68/$\delta_C$ 22.6 ppm) to account for this 'shoulder'.[3]

No signals were observed in either the 1D-$^{2}H$ NMR or 2D-$^{13}C$–$^{2}H$ COSY spectra of any of the crude extracts that

[‖] Some $^{2}H$ NMR spectra in Figure 4 also contain a weak deuterium singlet at $\delta_D$ 1.28 ppm, which is attributed to the natural abundance deuterium present in the methylene groups of long chain hydrocarbons in fatty acids (such compounds are always by far the most dominant components in the organic extracts of *A. annua*).

[**] The $^{13}C$ spectral projection shown on the horizontal axis of Figure 5 was acquired with $^{2}H$ decoupling ({$^{2}H$}–$^{13}C$) but *without* $^{1}H$ decoupling. This technique therefore tends to emphasize those signals arising from any $^{13}C$ resonance which is attached to $^{2}H$, such as that from the 15-position of **4a** and its metabolites, which appear as singlets; by contrast, isotopically-normal groups, in which carbon at natural abundance is attached to $^{1}H$, appear as much lower intensity multiplets. Although isotopically-normal $^{13}C$ peaks are therefore expected to be of lower intensity with {$^{2}H$}–$^{13}C$ decoupling, the {$^{2}H$}–$^{13}C$ decoupled spectral projection shown was, in fact, dominated by a triplet at $\delta_C$ 29.8 ($^{1}J_{CH}$=125 Hz) and a quartet at $\delta_C$ 14.1 ($^{1}J_{CH}$=124 Hz) ppm, both of which appear outside the expansion window. These peaks are associated with the long chain hydrocarbon components of the plant matrix which are several orders of magnitude more abundant in the crude plant extract than either compound **4a** or its metabolites.

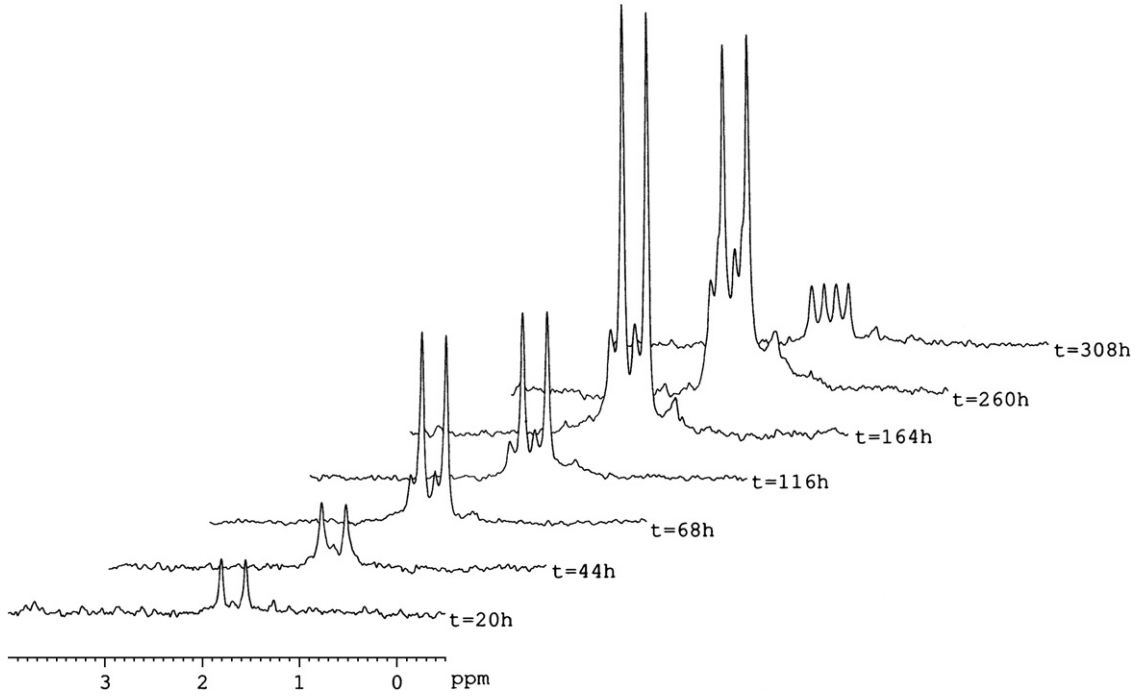



**Figure 4**. Expansion of the stacked plot of $^{2}$H NMR spectra from the seven crude $CH_2Cl_2$ extracts of the aerial compartment of *Artemisia annua* made after feeding [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**) via the root. These spectra demonstrate a substantial incorporation of **4a** ($\delta_D$ 1.68 ppm, d, $^{1}J_{CD}$=19.1 Hz) into the plant tissues and its gradual transformation into [15-$^{13}C^{2}H_3$]-3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (**17a**) ($\delta_D$ 1.81 ppm, d, $^{1}J_{CD}$=19.5 Hz). Each $^{2}$H NMR spectrum is shown in absolute intensity mode and variations in the peak heights therefore reflect variations both in the amount of precursor **4a** that had been fed (which was related, in turn, to the variation in the weight of each individual plant) and the amount of label that was recovered from the extract (which showed no consistent pattern with time).

would suggest the presence of any of the labeled metabolites [15-$^{13}C^{2}H_3$]-artemisinin (**1**), [15-$^{13}C^{2}H_3$]-arteannuin K (**7**), [15-$^{13}C^{2}H_3$]-arteannuin L (**8**) or [15-$^{13}C^{2}H_3$]-arteannuin M (**9**), which have been proposed previously as metabolites of **4** in *A. annua* (see Section 1).

### 2.5. Isolation and identification of labeled metabolites from the $CH_2Cl_2$ extracts of the aerial parts of *A. annua* that had been fed with [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (4a)

In order to ascertain the identities of the metabolites of **4a** which had been indicated in the previous section by their $^{13}$C and $^{2}$H NMR chemical shifts at the 15-position (definitive identification for $\delta_D$ 1.81/$\delta_C$ 19.9 ppm and $\delta_D$ 1.69/$\delta_C$ 19.6 ppm), the combined $CH_2Cl_2$ extracts of the aerial compartments of *A. annua* were subjected to repeated HPLC chromatographic separations (Section 4.6). This procedure resulted in the isolation of three labeled compounds, in addition to the labeled precursor **4a**. The structure and relative stereochemistry of the three novel metabolites, compounds **17a**–**19a** were all then established by the 2D-NMR experiments: HSQC, HMBC, $^{1}$H–$^{1}$H COSY, and NOESY (Table 1 and Fig. 6). All three metabolites were derived from the cadinane skeleton, and all retained the same five-membered lactone-ring, which was present in the precursor **4a**, with additional chemical modifications having occurred at either the C-3 or the C-9 position.

[15-$^{13}C^{2}H_3$]-3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (**17a**) was the most abundant metabolite to be isolated from HPLC separation and this compound was clearly responsible for the NMR signals which had been observed at $\delta_D$ 1.81 ppm/$\delta_C$ 19.9 ppm in both the $^{2}$H NMR time-course studies (Fig. 4) and in the $^{13}$C–$^{2}$H COSY spectrum of the combined crude plant extracts (Fig. 5). The NMR spectra for this metabolite were identical with those of the minor product, compound **17a**, which had been isolated from re-purification of synthetic **4a** just prior to commencement of the feeding experiment (Section 2.1). Because this same compound was presumed to have arisen in vitro by spontaneous autoxidation to a secondary allylic hydroperoxide which then underwent rearrangement and hydrolysis, as is shown in Figure 3, it was thus considered possible that the biogenesis of **17** in vivo in *A. annua* might also have occurred by such a non-enzymatic autoxidation mechanism, involving **11a** and **14a** as intermediates, as is suggested at the top of Scheme 1. However, it is impossible to discount the alternative possibility that compound **17a** might also have been formed enzymatically (and more directly) by a cytochrome $P_{450}$-mediated oxygenation at the 3-position, directed toward the α-face of compound **4a**.

[15-$^{13}C^{2}H_3$]-3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (**18a**) was isolated in a smaller amount than compound **17a** and appeared to be responsible for the resonances which had been observed at $\delta_D$ 1.69 ppm/$\delta_C$ 19.6 ppm in the $^{13}$C–$^{2}$H COSY spectrum of the combined crude plant extracts (Fig. 5). This metabolite has probably been formed by enzymatic acetylation of the 3α-hydroxyl group in compound **17a**, as is shown in Scheme 1. Although the structure of [15-$^{13}C^{2}H_3$]-3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (**18a**) was determined by 2D-NMR, as for all the other metabolites of **4a**, its structural elucidation was less rigorous

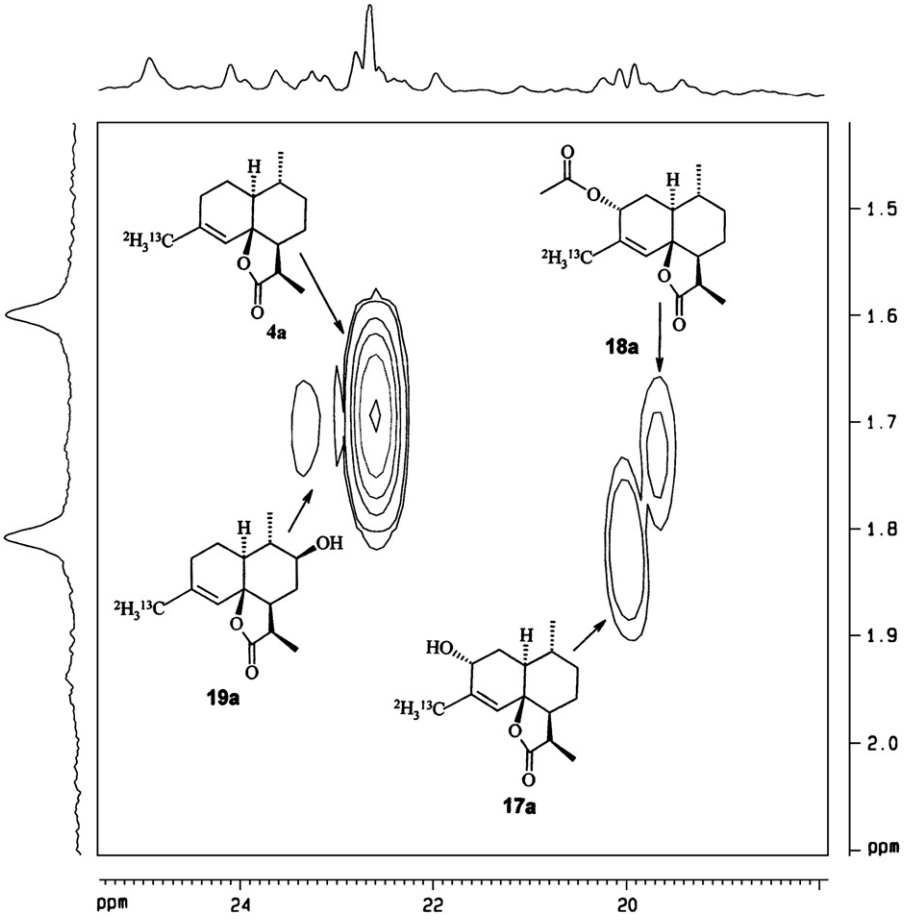


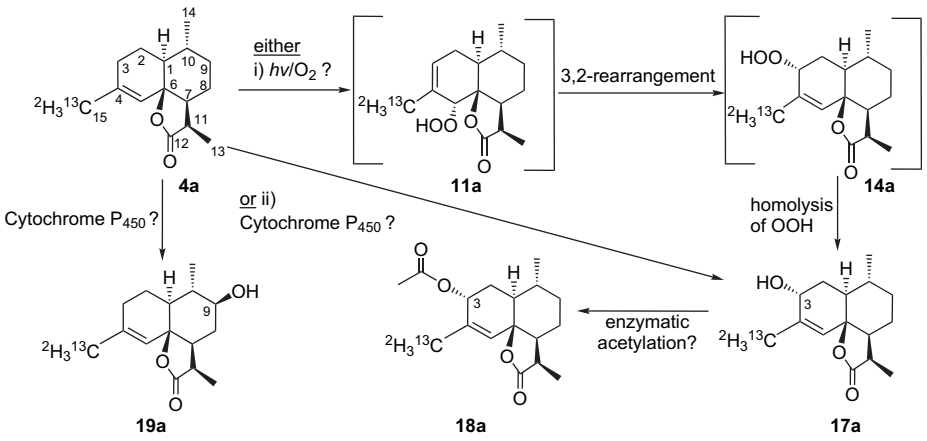

**Figure 5**. Expansion of the $^{13}C$–$^{2}H$ COSY NMR spectrum of the combined $CH_2Cl_2$ extracts from the aerial parts of *Artemisia annua* plants which had been fed with **4a**, showing the recovery both of the precursor **4a** and of its metabolites **17a** and **18a** (metabolite **19a** was also very tentatively identified by this technique).

than expected, as no direct correlation was observed in the HMBC spectrum from the acetyl substituent at C-3 to any of the protons in the decalin ring of **18a** or vice versa (Fig. 6)—even though this ester linkage was implied by the strong downfield shift of the H-3β proton in **18a** ($\delta_H$ 5.25 ppm) as compared with that of H-3β of the free alcohol

**Table 1**. $^{13}C$ and $^{1}H$ NMR data for the novel labeled compounds **16a** and **17a** isolated from the spontaneous in vitro autoxidation of [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyartennuin B (**4a**) and of compounds **17a**–**19a** isolated from the in vivo transformations of **4a** in *Artemisia annua* plants

| Position (mult.)[a] | $\delta_C$ | | | | $\delta_H$ | | | |
|---|---|---|---|---|---|---|---|---|
| | **16a** | **17a** | **18a** | **19a** | **16a** | **17a** | **18a** | **19a** |
| 1 (CH) | 46.6 | 41.0 | 41.9 | 45.0 | 1.29 | 1.56 | 1.49 | 1.27 |
| 2 ($CH_2$) | 31.7 | 31.0 | 28.0 | 20.7 | 2.33, 1.69 | 1.99, 1.97 | 1.98, 1.98 | 1.91, 1.75 |
| 3 (CH) | 71.0 | 67.6 | 69.5 | 30.9 ($CH_2$) | 4.06 | 4.08 | 5.25 | 2.15, 2.09 |
| 4 (C) | 144.2 | [b] | 129.2 | [b] | — | — | — | — |
| 5 (CH) | 124.4 | 125.0 | 127.3 | 121.5 | 5.71 | 5.75 | 5.88 | 5.64 |
| 6 (C) | 83.2 | 82.4 | 82.0 | [b] | — | — | — | — |
| 7 (CH) | 42.5 | 42.1 | 42.2 | 42.5 | 2.09 | 2.14 | 2.16 | 2.27 |
| 8 ($CH_2$) | 23.6 | 23.7 | 23.7 | 33.5 | [b, b] | 1.75, 1.21 | 1.73, 1.25 | 1.16, 1.99 |
| 9 ($CH_2$) | 31.7 | 32.3 | 32.2 | 73.8 (CH) | 1.66, 1.26 | 1.70, 1.09 | 1.70, 1.08 | 3.26 |
| 10 (CH) | 29.4 | 29.1 | 29.0 | 37.6 | 1.48 | 1.47 | 1.46 | 1.44 |
| 11 (CH) | 39.7 | 39.4 | 39.4 | 39.8 | 3.13 | 3.14 | 3.14 | 3.19 |
| 12 (C) | 178.8 | 179.1 | 178.8 | 178.7 | — | — | — | — |
| 13 ($CH_3$) | 9.4 | 9.4 | 9.4 | 9.4 | 1.15 | 1.16 | 1.16 | 1.18 |
| 14 ($CH_3$) | 19.7 | 19.4 | 19.4 | 14.0 | 0.95 | 0.96 | 0.90 | 1.05 |
| 15 ($CH_3$) | 19.4[c] | 19.9[c] | 19.6[c] | 22.5[c] | 1.82 | 1.81 | 1.69 | 1.67 |
| 16 (C) | — | — | 170.6 | — | — | — | — | — |
| 17 ($CH_3$) | — | — | 21.2 | — | — | — | 2.09 | — |

[a] Multiplicity determined from DEPT.
[b] Not resolved.
[c] $\delta_C$ Values reported for the 15-position are for the [15-$^{13}C^{2}H_3$]-isotopomer, i.e., the $^{13}C$ chemical shift of the isotopically-normal compound is expected to be ca. 0.9 ppm downfield from the resonance shown. See Section 4 for additional multiplicity and couplings in the $^{13}C$ spectrum due to $^{13}C$–$^{2}H$ couplings of these isotopically-labeled compounds.




**Scheme 1**. Proposed biogeneses of the labeled metabolites **17a**–**19a** which were isolated from the $CH_2Cl_2$ extracts of the aerial parts of *Artemisia annua* after feeding [15-$^{13}C^2H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**).

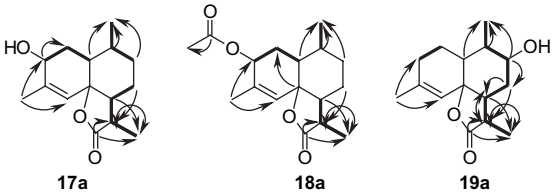



**Figure 6**. 2D-NMR correlations used in assigning the structures of the metabolites **17a**, **18a**, and **19a** (HMBC correlations are indicated by arrows from $^{13}C$ to $^{1}H$; $^{1}H$–$^{1}H$ COSY correlations are indicated by bold lines on the structure).

**17a** ($\delta_H$ 4.08 ppm) (Table 1). Fortunately, it was possible to obtain independent confirmation of the structure of **18a** by treating a sample of **17a** with acetic anhydride (Section 4.6.5), which resulted in a product with identical physical properties to those of **18a**.

The third and least abundant metabolite to be chromatographically purified from the feeding of **4a** to *A. annua* plants was [15-$^{13}C^2H_3$]-9β-hydroxy-dihydro-*epi*-deoxyarteannuin B (**19a**). Although the presence of this labeled natural product had been very tentatively suggested by the preliminary $^{13}C$–$^{2}H$ COSY analysis of the crude extract described in Section 2.4 (Fig. 5), its definitive identification was only possible following its isolation from the mixture. This is because hydroxylation at the 9-position of **19a** has little effect on either the $^{13}C$ or $^{2}H$ chemical shifts at the 15-position of this metabolite (see Table 1)—hence the presence of compound **19a** was largely obscured in both the 1D- and 2D-NMR spectra of the crude plant extracts by the much greater amount of the precursor **4a** that was present. There are no structural features presented by compound **4a** which would activate it toward spontaneous autoxidation at the 9-position (note that this is not the case for the 3-position), hence this metabolite must be formed enzymatically—most probably by the stereospecific action of a cytochrome $P_{450}$, which effects hydroxylation from the β-face at the 9-position of **4a**.

Finally, it should be noted that many other natural products were also isolated from the extensive HPLC separations which were performed in order to obtain metabolites **17a**–**19a** in sufficiently pure form for their physical characterization. Amongst these were the sesquiterpenes: artemisinin (**1**), arteannuin K (**7**), arteannuin L (**8**), and arteannuin M (**9**), all of which have been postulated previously as metabolites of **4** (see Section 1). However, no detectable $^{2}H$ NMR signal was observed in any of these natural products.

## 3. Conclusion

The observed transformations undergone in vivo by [15-$^{13}C^2H_3$]-dihydro-*epi*-deoxyarteannuin B (**4a**) in *A. annua* may be explained, in part, by the known spontaneous autoxidation chemistry of compound **4**[21] in vitro (see, for example, Fig. 3 for a possible biogenesis of compound **17a**). However, enzymatic transformations, such as the acetylation of **17a** and the hydroxylation of **4a** at the 9β-position, must be postulated in order to account for transformations to the metabolites **18a** and **19a**, respectively (Scheme 1). These in vivo experiments have also indicated that dihydro-*epi*-deoxyarteannuin B (**4**) seems to be neither a precursor to artemisinin (**1**) in *A. annua*, as had been claimed by Wang et al.,[16] nor an intermediate in the formation of arteannuins K–M (**7**–**9**), as has been more tentatively proposed by the authors.[8] Rather, the nature of the transformations identified—involving hydroxylation and acetylation at both the 3- and 9-positions of the decalin ring in **4**—are more suggestive of a degradative metabolism, which is designed to eliminate this compound from the plant.

In support of this conclusion, it should be noted that several other 3α- and 9β-hydroxylated[††] derivatives of sesquiterpene natural products from *A. annua* may also be viewed as being degradative in nature. Thus, there are four cadinane/amorphane sesquiterpenoids incorporating a 9β-hydroxyl group which have been described in previous studies of *A. annua*. Of these, 9β-hydroxyartemisinic acid[25] was isolated as its glycoside from the feeding of artemisinic acid to suspension cultures of *A. annua*; 9β-hydroxy-11-*epi*-artemisinin and

[††] Two 3β-hydroxyl derivatives of cadinane and amorphane sesquiterpene natural products from *A. annua* are also known: the 3β-hydroxyl derivative of artemisinic acid, which has been reported as its glycoside from plant tissue cultures of *A. annua*[25] and 3β-hydroxyarteannuin B from microbial transformation of arteannuin B.[14]

9β-hydroxy-11-*epi*-deoxyartemisinin were found as microbial metabolites of artemisitene (the 11,13-dehydro analogue of **1**),[26] and 9β-hydroxyarteether was obtained by the microbial oxidation of arteether[27] (a semi-synthetic derivative of artemisinin). The natural product 3α-hydroxydeoxyartemisinin (qinghaosu-IV) from *A. annua*[28,29] is perhaps formed in vivo by radical rearrangements of the peroxy bridge in artemisinin,[30–33] although it is also known as a microbial metabolite of artemisinin.[27,28,34]

Since dihyro-*epi*-deoxyarteannuin B (**4**) appears not to be involved in the biosynthesis of **1**, it can be concluded that the pathway which has been proposed for the conversion of **3** into **1**, involving this compound as an intermediate (i.e., the uppermost dashed arrow in Fig. 1), can now be dismissed. If the spontaneous autoxidation of dihydroartemisinic acid[2–9] to the hydroperoxide **3** is indeed the late committed step in the dominant biosynthetic route to artemisinin in vivo,[8] three possibilities then remain: either, the biosynthesis of **1** proceeds via further enzymatic transformations of dihydroarteannuin B (**5**), as has been proposed by Jain et al,[17] or, it proceeds via further—probably enzymatic—transformations of **6**,[8] or, the conversion of **3** into **1** occurs spontaneously by Hock cleavage, as has been observed in vitro[12] (i.e., the lower dashed arrow in Fig. 1). Clearly, further feeding experiments, in which stable isotope-labeled forms of **5** or **6** are administered to intact plants of *A. annua*, would help to resolve this issue. In addition, it would be interesting to perform experiments in the hydrophobic environment of a plant trichome, which are designed to detect whether the direct Hock cleavage of **3** can lead directly to compound **1** via a transient enol, as is shown at the bottom of Figure 1.

## 4. Experimental

### 4.1. General information

All $^{1}H$ and $^{13}C$ NMR experiments were recorded on either a Bruker AMX 400, DRX 500 or AV 600 instrument. Chemical shifts are expressed in parts per million ($\delta$) relative to TMS as internal reference. Proton chemical shifts, multiplicities, coupling constants, and integrals reported in this section are those which were clearly resolved in 1D-$^{1}H$ NMR without recourse to 2D-NMR analysis (see Table 1 in the main text for full assignments which were made by 2D-NMR). $^{2}H$ NMR spectra[‡‡] were recorded in $CHCl_3$ solution containing $C_6D_6$ (10 μl/100 ml) which was used both as an internal reference ($\delta_D$ 7.43 ppm) and as internal calibration standard for estimating the amounts of $^{2}H$-labeled metabolites in solution (the ratio of the $^{2}H$ integral for the benzene-$d_6$ internal standard to the $^{2}H$ integral for **4a** and the metabolite **17a**, which were resolved in the crude plant extract, was routinely used to estimate the amount of $^{2}H$ label which was present in such extracts). The chemical shift of this reference compound did not interfere with the products of metabolism of dihydro-*epi*-deoxyarteannuin B, for which $^{2}H$ signal was normally observed only in the aliphatic region of the spectrum ($\delta_D$ 1.0–2.5 ppm)—see Section 2.4.

$^{13}C$–$^{2}H$ COSY NMR[8,22,23] spectra were acquired with an AV 600 MHz NMR spectrometer employing some modifications to the procedures which have been described previously for a Bruker DRX 500 spectrometer[22,23]—e.g., the $^{2}H$ 'hard' 90° pulses (70 μs/−6 dB) and composite decoupling pulses (Waltz16; 230 μs/8 dB) which are required for the $^{13}C$–$^{2}H$ COSY experiment were now created by a '2H-TX' board, which was incorporated as standard on the AV 600 spectrometer. Because this version of the 2H-TX board is essentially a gated 20 W amplifier and interface—converting the lock channel circuitry from its normal function to a transmit/receiver mode during pulses, but allowing the normal operation of the lock at all other times—it was possible to prepare samples for $^{13}C$–$^{2}H$ COSY spectroscopy in $CDCl_3$, rather than $CHCl_3$ solution, as previously reported,[22,23] and therefore to continue to auto-shim the sample over prolonged periods of acquisition time. In addition, since the BBO probe for the 600 MHz spectrometer had been designed for $^{2}H$ excitation, it was possible to use conventional 5 mm NMR tubes to prepare the $^{13}C$–$^{2}H$ COSY sample (2.5 mm microtubes had been employed previously for use with a dual probe at 500 MHz).[22,23]

HSQC, HMBC, $^{1}H$–$^{1}H$ COSY, and NOESY spectra were recorded with 2048 data points in $F_2$ and 256 data points in $F_1$. High-resolution MS were recorded in EI mode at 70 eV on a Finnigan-MAT 95 MS spectrometer. IR spectra were recorded in $CHCl_3$ on a Shimadzu FT-IR-8201 PC instrument. HPLC separations were performed using a Varian chromatogram equipped with RI star 9040 and UV 9050 detectors and a YMC diol column (20 mm×25 cm), flow rate 8 ml/min. Optical rotations were measured by a Perkin–Elmer 343 polarimeter (Na 589 nm). $[\alpha]_D$ values are given in $10^{-1}$ deg cm$^2$ g$^{-1}$ and $CHCl_3$ was used as a solvent.

### 4.2. Synthesis and re-purification of labeled [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (4a)

See Ref. 21 for details of the synthesis of **4a** and the previously reported HPLC re-purification of a sample which had been stored for one year, yielding compounds **7a** and **10a**–**15a**, in addition to **4a**. In the current study, approximately 80 mg of **4a** was synthesized by the same procedure and stored in the freezer for a slightly longer time. Re-purification by HPLC yielded compounds **16a** and **17a** in addition to the compounds described previously.[21] Purified **4a** was then used immediately in the feeding experiments, which are described in Section 4.4.

**4.2.1. [15-$^{13}C^{2}H_3$]-dihydro-*epi*-deoxyarteannuin B (4a).** Oil (45 mg, $t_R$ 13.4 min, 13% EtOAc/*n*-hexane) (see also Refs. 3 and 12 for physical properties, as well as Section

[‡‡] In order to acquire $^{2}H$ NMR spectra, the broadband nucleus was set to the $^{2}H$ frequency and the lock cable was physically disconnected from the probe of the NMR spectrometer, so as to prevent any leakage of the lock signal into the acquisition channel. Since it then became impossible to shim the sample, normal practice was to have first shimmed an NMR tube of the same manufacture, containing exactly the same volume of $CDCl_3$ solution (0.6 ml), just prior to the acquisition of each $^{2}H$ NMR spectrum. Using this technique, the linewidth at half height for most $^{2}H$ NMR spectra was found to be fairly constant (between 2.5 and 3.5 Hz); the major contribution to line broadening in $^{2}H$ NMR spectra must then be from the unavoidable effects of quadrupolar relaxation in $^{2}H$ NMR spectroscopy,[35,36] rather than from magnetic inhomogeneities which were associated with poor shimming.

4.6.1): $^1$H NMR ($\delta$, $CDCl_3$) ppm: 5.63 (1H, d, $J$=6.2 Hz, $^3J_{CH}$ coupling to C-15, H-5), 3.15 (1H, dq, $J$=7.1, 7.1 Hz, H-11), 2.11 (H-7), 2.10 (H-3α), 2.06 (H-3β), 1.89 (H-2α), 1.73 (H-8α), 1.69 (H-2β), 1.68 (H-15) is not seen because of deuteriation, 1.67 (H-9β), 1.42 (H-10), 1.26 (H-1), 1.18 (H-8β), 1.14 (3H, d, $J$=7.1 Hz, H-13), 1.06 (H-9α), 0.93 (3H, d, $J$=6.5 Hz, H-14); $^{13}$C NMR ($\delta$, $CDCl_3$) ppm: 179.4 (C-12), 142.2 (d, $J$=42.9 Hz, $^1J_{CC}$, C-4), 121.8 (C-5), 83.2 (d, $J$=4.0 Hz, $^3J_{CC}$, C-6), 46.6 (C-1), 42.8 (C-7), 39.7 (C-11), 32.4 (C-9), 30.8 (d, $J$=3.0 Hz, $^2J_{CC}$, C-3), 29.6 (C-10), 23.8 (C-8), 23.5 (s, 1% for 15-$^{13}CH_3$), 23.2 ((1:1:1) t, $J$=19.2 Hz, $^1J_{CD}$, 6% for 15-$^{13}CH_2{}^2H$), 22.9 ((1:2:3:2:1) quin., $J$=19.2 Hz, $^1J_{CD}$, 10% for 15-$^{13}CH^2H_2$), 22.6 ((1:3:6:7:6:3:1) sept., $J$=19.2 Hz, $^1J_{CD}$, 83% for 15-$^{13}C^2H_3$), 21.0 (d, $J$=2.8 Hz, $^3J_{CC}$, C-2), 19.6 (C-14), 9.4 (C-13); $^2$H NMR ($\delta$, $CHCl_3$) ppm: 1.68 (d, $J$=19.2 Hz, $^1J_{CD}$); HREIMS—see Ref. 21.

**4.2.2. [15-$^{13}C^2H_3$]-3β-hydroxy-dihydro-*epi*-deoxyarteannuin B (16a).** Oil (1.5 mg, 2%). $^1$H NMR ($\delta$, $CDCl_3$) ppm: 5.71 (1H, d, $J$=6.0 Hz, $^3J_{CH}$ coupling to C-15, H-5), 4.06 (1H, dd, $J$=9.7, 6.3 Hz, H-3), 3.13 (1H, dq, $J$=6.3, 7.1 Hz, H-11), 2.33 (1H, ddd, $J$=12.9, 6.3, 2.2 Hz, H-2α), 1.82 (H-15) is not clearly seen because of deuteriation, 1.15 (3H, d, $J$=7.1 Hz, H-13), 0.95 (3H, d, $J$=6.6 Hz, H-14)—see also Table 1; $^{13}$C NMR—see Table 1; $^2$H NMR ($\delta$, $CDCl_3$) ppm: 1.82 (d, $J$=19.1 Hz, $^1J_{CD}$).

**4.2.3. [15-$^{13}C^2H_3$]-3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (17a).** Oil (2 mg, 2%). See Section 4.6.2 for physical properties.

### 4.3. *A. annua* plants

*A. annua* Plants were grown outdoors from seed (seeds were supplied by the NCCPG *Artemisia* collection, Cambridge, UK and taxonomically verified specimens are held by the collection). Plants were used for this feeding experiment after approximately three months, when mature and beginning to come into flower.

**4.3.1. Preparation of a feeding solution of 4a in a medium incorporating Triton®.** Initial studies were directed toward determining the concentration of Triton® X-100 (purchased from Sigma) which would cause no discernible physical injury to intact *A. annua* plants. Thus, several experiments were conducted in which *A. annua* plants were immersed by the root for variable periods of time in aqueous solutions containing differing concentrations of Triton®, but lacking the feeding precursor dihydro-*epi*-deoxyarteannuin B. From these experiments, it was concluded that small plants (3–5 g fresh weight) could remain alive and healthy for a period of up to two weeks under hydroponic conditions after having been immersed by the root in a 0.1% aqueous solution of Triton® (3 ml) for a period of 6 h. This concentration of Triton® was therefore used for administering **4a** to *A. annua* in the feeding experiment which is described in Section 4.4.

NMR analysis of the plant tissues that had been extracted by $CH_2Cl_2$ during these preliminary experiments showed that, under all conditions tested, the characteristic $^1$H NMR signals for Triton® always remained associated with the root compartment, and were never detected in the aerial compartment.[§§] It therefore seemed quite likely that although Triton® might be adsorbed onto the root surface it was not assimilated by the tissues of *A. annua* (and it was certainly not translocated within the plant). By contrast, plants cut at the root, and stem-fed with solutions containing Triton® died within a few days and $^1$H NMR analysis consistently indicated a significant incorporation of Triton® into all of the plant tissues. Hence the administration of an aqueous solution of compound **4a** containing Triton® via the cut stem was not considered to be a viable feeding method when conducting prolonged metabolic studies.

The maximum solubility of **4a** in a 0.1% aqueous solution[¶¶] of Triton® was found to be ca. 1 mg/ml (after ultrasonification for 1 h).[‖‖] Hence, the stock solution used in the feeding experiments which are described below consisted of 20 mg of compound **4a** dissolved in 30 ml of an aqueous solution containing 0.1% Triton®.

### 4.4. Root-feeding of 4a to intact *A. annua* plants

The roots of seven intact *A. annua* plants (grown in pots) were cleaned free of soil and the plants were weighed individually. Each plant was then immersed by its root in an individual vial containing the feeding solution which is described in Section 4.3.1. The size and weights of the plants used in this experiment were quite variable (between 5 and 20 g) and approximately 1 ml of feeding solution was administered for each 2.5 g of fresh plant material. The plants were exposed to a strong light (500 W bulb) and a constant flow of air (from a nearby fan) in order to promote rapid uptake of the feeding solution via the transpiration stream. Uptake of the labeled precursor by the intact plants was usually completed within 4–6 h of the commencement of feeding. Plants were then kept alive outdoors for a period of up to two weeks under hydroponic conditions, which were maintained by continually replenishing the feeding vial with deionized water. The ambient temperature was 23–27 °C over the course of the experiment and the skies were mostly overcast.

**4.4.1. Extraction procedures.** Extracts of the seven individual plants were made at 20, 44, 68, 116, 164, 260, and 308 h after the termination of feeding. The total plant weight at extraction was found to be between 65 and 80% of the initial weight that had been determined before feeding; this loss in weight may indicate some dehydration as a result of the forcing conditions which were employed in order to stimulate transpiration. Each plant was first divided into a root portion and an aerial portion (which included stem, leaves, and flowers) and these two compartments were then extracted separately by grinding to a fine powder under liquid

[§§] Characteristic $^1$H NMR peaks for Triton® that were observed in crude extracts of the root compartment of *A. annua* plants were $\delta_H$ ($CDCl_3$) ppm: 7.25 (2H, d, $J$=9.5 Hz), 6.82 (2H, d, $J$=9.5 Hz), 4.11 (2H, t, $J$=4.8 Hz), 3.84 (2H, t, $J$=4.8 Hz), 3.72 (2H, br s), 3.65 (ca. 20H, br s), 3.61 (2H, br s), 2.08 (1H, br s), 1.69 (2H, s), 1.33 (6H, s), 0.70 (9H, s).

[¶¶] Although the solubility of **4a** was much higher in a 0.5% Triton® solution (up to 3 mg/ml), this concentration of Triton had previously been found to be somewhat injurious to the plants.

[‖‖] $^1$H NMR analysis showed no changes in the chemical composition of **4a** following ultrasonification.

$N_2$, followed by immersion in $CH_2Cl_2$ (ca. 20 ml solvent/1 g plant material) in a container wrapped in aluminum foil (in order to exclude light, and prevent any spontaneous autoxidation such as that shown in Fig. 3). The extraction vessels were left to stand overnight, then filtered free of plant particulates, and the solvent was dried ($MgSO_4$) and removed in vacuo to yield the crude plant extract. It was generally found that the efficiency of extraction was 1.5–2% (w/w) for the aerial compartment while the efficiency for the root compartment was around 0.2% (w/w). All the plant residues were then re-extracted by MeOH using similar procedures to those described above, but no significant recovery of $^2H$ label was noted for any of the MeOH extracts and these samples are not discussed further (the efficiency of extraction in MeOH was 2–5% (w/w) for both aerial and root compartments). Crude $CH_2Cl_2$ extracts of each plant were analyzed *immediately* after extraction (in order to minimize the possibility of autoxidation) by both $^2H$ and $^1H$ NMR.

### 4.5. $^2H$ NMR analysis of the crude $CH_2Cl_2$ extracts from the aerial parts of *A. annua* which had been fed with 4a

The initial analysis of each individual *A. annua* plant that had been fed with **4a** was made by $^2H$ NMR spectroscopy of the corresponding crude $CH_2Cl_2$ extract which was dissolved in 0.6 ml of $CHCl_3$, containing benzene-$d_6$, as reference and calibration standard (see Section 4.1). The extent of recovery of $^2H$ label from each $CH_2Cl_2$ extract was calculated by comparing the ratio of the total integral in the aliphatic region of each $^2H$ NMR spectrum (1.0–2.5 ppm) to that of the internal calibration standard, $C_6D_6$ at $\delta_D$ 7.43 ppm,*** as described in Section 4.1.

#### 4.5.1. $^{13}C$–$^2H$ COSY NMR analysis of the crude $CH_2Cl_2$ extracts from the aerial parts of *A. annua* which had been fed with 4a.

The crude $CH_2Cl_2$ extracts from all seven plant extracts were combined into a single sample (656 mg) which was re-dissolved in ca. 0.6 ml $CDCl_3$ and analyzed by $^{13}C$–$^2H$ COSY spectroscopy at 600 MHz (see Section 4.1).

### 4.6. Separation of labeled metabolites from the $CH_2Cl_2$ extracts of the aerial parts of *A. annua* which had been fed with 4a

All the $CH_2Cl_2$ extracts from the aerial compartments (stem, leaf, and flower) of the seven plants of *A. annua* which had been fed with **4a** were combined (656 mg) and subjected to a 'preliminary' HPLC separation (18% EtOAc/*n*-hexane) from which 30 fractions were collected over a 60 min period. A total of seven injections were made—each injection containing approximately 100 mg of the crude plant material—and the HPLC column was 'washed' with 50% EtOAc/*n*-hexane/1% AcOH between injections, resulting in an additional 'polar residue' fraction, collected from the combined washings. Only those individual fractions from the 'preliminary' HPLC separation which showed a doublet signal in $^2H$ NMR spectroscopy (i.e., fractions at $t_R$=12–13 min for **4a**, $t_R$=17–18 min for **18a**, and $t_R$=51–53 min for **17a**), were then re-subjected to further repetitive HPLC separations in order to obtain a sample of a pure labeled metabolite which was characterized by 2D-NMR spectroscopic analysis, as well as by other physical techniques. The 'polar residue' fraction, obtained from washing the HPLC column during the 'preliminary' separation, was also re-subjected to further repetitive HPLC separations (50% EtOAc/*n*-hexane/1% $CH_3COOH$): only one fraction was found to exhibit a doublet resonance in $^2H$ NMR spectroscopy, corresponding to compound **19a**, which was isolated from further purification of this fraction.

#### 4.6.1. [15-$^{13}C^2H_3$]-dihydro-*epi*-deoxyarteannuin B (4a)/dihydro-*epi*-deoxyarteannuin B (4).

Oil (3.8 mg, $t_R$ 28.1 min, 2.4% EtOAc/*n*-hexane). See Refs. 3, 11, and 21 and Section 4.2.1 for physical properties other than MS. HREIMS *m/z* (rel int.): 238.1838 [$M^+$, $C_{14}{}^{13}C_1H_{19}{}^2H_3O_2$ requires 238.1842] (30), 237 (5), 236 (6), 235 (4), 234 (10), 194 (85), 193 (14), 192 (34), 191 (14), 190 (38), 165 (100), 164 (29), 163 (21), 162 (15), 161 (39).

#### 4.6.2. [15-$^{13}C^2H_3$]-3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (17a)/3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (17).

Oil (1.0 mg, $t_R$ 15.5 min, 35% EtOAc/*n*-hexane). $[\alpha]_D$ +41.2 (*c* 0.2, $CHCl_3$); IR $\nu_{max}$ ($CHCl_3$): 3400 (br), 3015, 2926, 1759, 1456 $cm^{-1}$; $^1H$ NMR ($\delta$, $CDCl_3$) ppm: 5.75 (1H, d, *J*=6.1 Hz, $^3J_{CH}$ coupling to C-15), 4.08 (1H, ddd, *J*=4.2, 2.2, 2.2 Hz), 3.14 (1H, dq, *J*=6.3, 7.0 Hz), 1.16 (3H, d, *J*=7.0 Hz), 0.96 (3H, d, *J*=6.5 Hz)—see also Table 1; $^{13}C$ NMR ($\delta$, $CDCl_3$) ppm: 19.9 ((1:3:6:7:6:3:1) sept., *J*=19.3 Hz, $^1J_{CD}$, C-15)—see Table 1 for other resonances; $^2H$ NMR ($\delta$, $CHCl_3$) ppm: 1.81 (d, *J*=19.3 Hz, $^1J_{CD}$); HREIMS *m/z* (rel int.): 254.1797 [$M^+$, $C_{14}{}^{13}C_1H_{19}{}^2H_3O_3$ requires 254.1791] (12), 250.1563 [$M^+$, $C_{15}H_{22}O_3$ requires 250.1569] (2), 236 (10), 181 (100), 166 (65).

#### 4.6.3. [15-$^{13}C^2H_3$]-3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (18a)/3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (18).

Oil (0.4 mg, $t_R$ 31.8 min, 8% EtOAc/*n*-hexane). $[\alpha]_D$ +47.3 (*c* 0.09, $CHCl_3$); IR $\nu_{max}$ ($CHCl_3$): 3022, 2930, 2856, 1761, 1734 $cm^{-1}$; $^1H$ NMR ($\delta$, $CDCl_3$) ppm: 5.88 (1H, d, *J*=6.0 Hz, $^3J_{CH}$ coupling to C-15), 5.25 (1H, m), 3.14 (1H, dq, *J*=7.0, 6.9 Hz), 2.16 (1H, ddd, *J*=10.6, 7.0, 6.2 Hz), 2.09 (3H, s), 1.16 (3H, d, *J*=6.9 Hz), 0.90 (3H, d, *J*=6.3 Hz)—see also Table 1; $^{13}C$ NMR ($\delta$, $CDCl_3$) ppm: 19.6 ((1:3:6:7:6:3:1) sept., *J*=19.2 Hz, $^1J_{CD}$, C-15)—see Table 1 for other resonances; $^2H$ NMR ($\delta$, $CHCl_3$) ppm: 1.69 (d, *J*=19.2 Hz, $^1J_{CD}$); HREIMS *m/z* (rel int.): 296.1896 [$M^+$, $C_{16}{}^{13}C_1H_{21}{}^2H_3O_4$ requires 296.1897] (1), 295.1838 [$M^+$, $C_{16}{}^{13}C_1H_{22}{}^2H_2O_4$ requires 295.1834] (0.1), 294.1769 [$M^+$, $C_{16}{}^{13}C_1H_{23}{}^2HO_4$ requires 294.1771] (0.01), 254 (60), 236.1683 [$M^+-CH_3COOH$, $C_{14}{}^{13}C_1H_{17}{}^2H_3O_2$ requires 236.1685] (16), 192 (10), 181 (100), 163 (25).

#### 4.6.4. [15-$^{13}C^2H_3$]-9β-hydroxy-dihydro-*epi*-deoxyarteannuin B (19a)/9β-hydroxy-dihydro-*epi*-deoxyarteannuin B (19).

Oil (0.2 mg, $t_R$ 20.7 min, 35% EtOAc/*n*-hexane/1% AcOH). $^1H$ NMR ($\delta$, $CDCl_3$) ppm: 5.64 (1H, d, *J*=6.2 Hz, $^3J_{CH}$ coupling to C-15), 3.26 (1H, ddd, *J*=11.2, 11.2, 3.0 Hz), 3.19 (1H, dq, *J*=7.0, 6.5 Hz), 1.18 (3H, d, *J*=6.5 Hz), 1.05 (3H, d, *J*=6.2 Hz)—see also Table 1; $^{13}C$ NMR ($\delta$, $CDCl_3$) ppm: 22.6 ((1:3:6:7:6:3:1) sept., *J*=19.3 Hz, $^1J_{CD}$, C-15)—see Table 1 for other resonances; $^2H$ NMR ($\delta$, $CHCl_3$) ppm: 1.68 (d, *J*=19.3 Hz, $^1J_{CD}$).

*** These $^2H$ NMR spectra also showed a smaller peak at $\delta_D$ 7.28 ppm, due to natural abundance $^2H$ present in the $CHCl_3$ solvent.

**4.6.5. Conversion of [15-$^{13}C^{2}H_3$]-3α-hydroxy-dihydro-*epi*-deoxyarteannuin B (17a) to [15-$^{13}C^{2}H_3$]-3α-acetoxy-dihydro-*epi*-deoxyarteannuin B (18a).** To a solution of compound **17a** (1.3 mg, $5.2\times10^{-6}$ mol, obtained from the semi-preparative HPLC separation of the mixture from the spontaneous autoxidation of **4a**—see Section 4.2.3) in acetic anhydride (0.5 ml) was added $ZnCl_2$ (3 mg, $2.2\times10^{-5}$ mol). The reaction mixture was stirred at room temperature for 2 h and completion of the reaction was monitored by TLC. The organic phase was then washed successively by 20% $NaHCO_3$ (2×2 ml) and brine (2×2 ml), dried ($MgSO_4$), and the organic solvent removed in vacuo to yield compound **18a** (1.1 mg, $3.77\times10^{-6}$ mol, 85%) without the need for further purification. Physical properties of **18a** which were obtained from synthesis were identical with those of the metabolite obtained from *A. annua* (Section 4.6.3).

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