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In vivo transformations of dihydroartemisinic acid in *Artemisia annua* plants

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Abstract—[15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**2a**) and [15-$C^{2}H_3$]-dihydroartemisinic acid (**2b**) have been fed via the root to intact *Artemisia annua* plants and their transformations studied in vivo by one-dimensional ^{2}H NMR spectroscopy and two-dimensional $^{13}C-^{2}H$ correlation NMR spectroscopy ($^{13}C-^{2}H$ COSY). Labelled dihydroartemisinic acid was transformed into 16 12-carboxy-amorphane and cadinane sesquiterpenes within a few days in the aerial parts of *A. annua*, although transformations in the root were much slower and more limited. Fifteen of these 16 metabolites have been reported previously as natural products from *A. annua*. Evidence is presented that the first step in the transformation of dihydroartemisinic acid in vivo is the formation of allylic hydroperoxides by the reaction of molecular oxygen with the $\Delta^{4,5}$-double bond in this compound. The origin of all 16 secondary metabolites might then be explained by the known further reactions of such hydroperoxides. The qualitative pattern for the transformations of dihydroartemisinic acid in vivo was essentially unaltered when a comparison was made between plants, which had been kept alive and plants which were allowed to die after feeding of the labelled precursor. This, coupled with the observation that the pattern of transformations of **2** in vivo demonstrated very close parallels with the spontaneous autoxidation chemistry for **2**, which we have recently demonstrated in vitro, has lead us to conclude that the main 'metabolic route' for dihydroartemisinic acid in *A. annua* involves its spontaneous autoxidation and the subsequent spontaneous reactions of allylic hydroperoxides which are derived from **2**. There may be no need to invoke the participation of enzymes in any of the later biogenetic steps leading to all 16 of the labelled 11,13-dihydro-amorphane sesquiterpenes which are found in *A. annua* as natural products.

1. Introduction

The Chinese plant *Artemisia annua* L. has been the subject of intensive phytochemical investigation over the past two decades following the discovery of the anti-malarial amorphane sesquiterpene artemisinin (qinghaosu) (**1**).[1] Some 47 amorphane and cadinane sesquiterpenes are currently reported from this species in the literature,[2–6] 35 of which contain a 12-carboxy group—the majority of these amorphane and cadinane sesquiterpenes are currently known in both their 11,13-dihydro and 11,13-dehydro forms (see compounds **1**–**28** in Figure 1, for examples). Although the biosynthesis of the amorphane/cadinane skeleton undoubtedly proceeds from mevalonic acid in *A. annua*,[7–10] and requires the participation of those enzymes which are normally associated with sesquiterpene biosynthesis (some of which have now been characterized from this species),[11,12] there is much more uncertainty regarding the manner of the subsequent skeletal rearrangement (involving carbon–carbon cleavage at C-4/C-5) which ultimately gives rise to the 1,2,4-trioxane system of artemisinin (**1**).[13]

A number of previous attempts have been made to delineate the biosynthetic pathway to the unique 1,2,4-trioxane ring in **1**, and experimental evidence has been presented that several of the natural products shown in Figure 1 may be late biosynthetic intermediates *en route* to artemisinin in either their 11,13-dihydro or 11,13-dehydro forms. However, it is clear that only some of these proposals can be correct. Thus, although all investigators seem to concur that either dihydroartemisinic acid (**2**)[14,15] or its 11,13-dehydro analogue artemisinic acid (arteannuic acid) (**3**)[15,17–22] are advanced precursors *en route* to artemisinin, there are several differing, and sometimes directly conflicting, views as to exactly how their transformation into **1** occurs. (It should be noted that compound **3** is also hydroxylated and derivatized as glycosides by plant tissue cultures of *A. annua*, in what appears to be a degradative pathway.)[16] For example, while both Nair and Basile[23,24] and Jain et al.[25] have found experimental evidence that arteannuin B (**4**), or its 11,13-dihydro analogue, dihydroarteannuin B (**5**), can be

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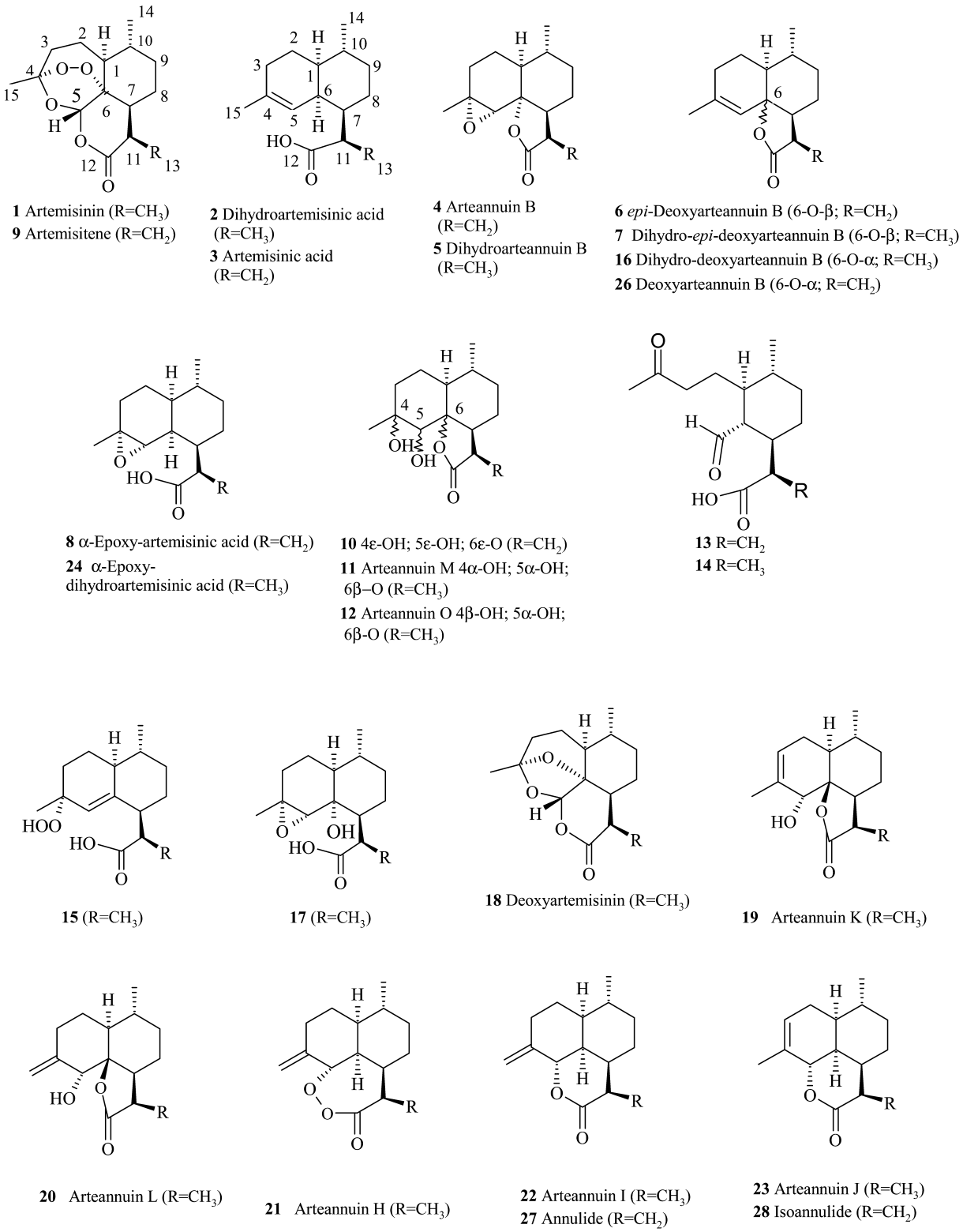


Figure 1. 12-Carboxy-amorphanes and cadinanes discussed in the text which have been reported as natural products from *A. annua*. Note that the majority of these compounds are known in both their 11,13-dihydro and 11,13-dehydro forms.

converted into artemisinin (**1**) by cell-free extracts, Wang et al. have noted that compound **4** was not a precursor of **1**,[15] but suggested rather that *epi*-deoxyarteannuin B (**6**) and dihydro-*epi*-deoxyarteannuin B (**7**) are the true intermediates in the conversion of **2/3** to **1**.[26] They also rejected the 11,13-dehydro natural product, α-epoxy-artemisinic acid (**8**),[27] as a biogenetic precursor of **1**,[15] and it has been proposed by these (and other) authors that the 11,13-dehydro-amorphane artemistene (**9**) may, in fact, be the immediate precursor of **1**.[15,23,28] Dihydroxy-amorphanes, such as compound **10**[29] (and by logical extension its 11,13-dihydro analogues, arteannuins M (**11**)[2,5] and O (**12**)),[5] have

also been implicated as possible biogenetic precursors, which might be converted to **1** via Grob fragmentation to an enolic intermediate which is tautomeric with the *seco*-amorphane **13**[29] (or its 11,13-dihydro analogue **14**),[6] but there is as yet no experimental evidence to support this.

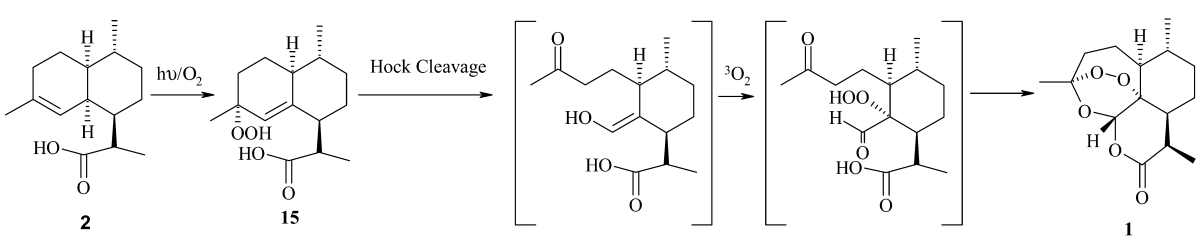
On the other hand, there are now quite a large number of studies which have shown that dihydroartemisinic acid (**2**)/artemisinic acid (**3**) can be transformed into artemisinin (**1**)/artemisitene (**9**) in the chemistry laboratory by photosensitized oxygenation. The initial product of this reaction has been identified as the tertiary allylic hydroperoxide **15**[2] (or its 11,13-dehydro equivalent), which is in turn readily converted to artemisinin (or its 11,13-dehydro equivalent, compound **9**) by reagents such as acid and/or copper (II) in the presence of atmospheric oxygen.[30–38] It has been proposed by Haynes et al. that the mechanism for this chemical conversion involves an enolic intermediate,[33–35] such as that shown in Scheme 1, and we have recently provided convincing evidence from detailed low temperature 2D NMR studies[39] for both the structure and participation of this same intermediate in the mechanism of the spontaneous autoxidation of **2**, which occurs at a much slower rate. The possible relevance to the biogenesis of artemisinin (**1**) in vivo of this 'preparative' pathway for the chemical synthesis of **1** from **2** has been highlighted within the past few years by the isolation of the tertiary allylic hydroperoxide **15** as a natural product from *A. annua*.[40,41] We have since shown that the spontaneous autoxidation of **2** also proceeds via compound **15**, in precisely the same manner which had previously been proposed for the 'chemical' transformation of **2** to **1** by photosensitized oxygenation and treatment with acid/Cu(II) (Scheme 1), but that, in actuality, it requires nothing more[†] than the presence of light and molecular oxygen.[39,43] We have, in addition, shown that compound **15** can also be spontaneously transformed in vitro into dihydroarteannuin B (**5**),[2,39,43] dihydro-*epi*-deoxyarteannuin B (**7**),[2,39] the *seco*-cadinane **14**[6,39] and several other natural products which have previously been obtained as natural products from *A. annua* (Fig. 1).

2. Results and discussion

We set out first to confirm that dihydroartemisinic acid (**2**) is a late biogenetic precursor of artemisinin (**1**) in *A. annua*, as seems to be generally accepted, and secondly to clarify the pathway by which the remarkable transformation of **2** into **1** occurs in vivo. In order to achieve this, we have fed isotopically-labelled dihydroartemisinic acid (either [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**2a**), [15-$C^{2}H_3$]-dihydroartemisinic acid (**2b**) or [15-$^{13}CH_3$]-dihydroartemisinic acid (**2c**))[‡] to intact *A. annua* plants via the root. This is one of the most realistic scenarios for studying transformations of **2** in vivo that can be envisaged and we believe it to be an improvement on all previous biosynthetic experiments with *A. annua*, which have routinely employed cell-free extracts. Feeding the labelled precursor to the plant by the intact root is especially favourable when it is considered important to avoid any possibility for the introduction of artifacts which might be created by the spontaneous oxygenation of **2** through exposure to the atmosphere, such as might easily occur during the extensive manipulations of the biological source material which are required when preparing and studying cell-free extracts (see Refs. 39 and 43 and the companion paper for a discussion of the unexpected readiness with which the precursor **2** undergoes such spontaneous autoxidation reactions both in organic solution and on storage).

2.1. Determination of the optimum method for feeding of [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (2a) to *A. annua* plants

The solubility of dihydroartemisinic acid in deionized water was quite poor, and in order to standardize the conditions for feeding aqueous solutions of either compounds **2a**, **2b** or **2c** to *A. annua* plants, solutions of these labelled precursors were routinely prepared in TRIS buffer (at pH 8.2), which effectively fixed the carboxylate group at the 12-position of **2** in the more soluble conjugate base form, thereby enhancing its aqueous solubility. TRIS was found to be non-injurious to plants (by visual inspection, at least) at the concentrations which were required to provide an effective buffering capacity and for the quantities of dihydroartemisinic acid which were fed to individual plants in each of the experiments described in Sections 2.2–2.4. The ability of *A. annua* plants to assimilate the labelled precursor, either when cut just above the root and immersed by the cut stem in a buffered feeding solution



Scheme 1. Mechanism for the spontaneous conversion of dihydroartemisinic acid (**2**) to artemisinin (**1**) in vitro via the tertiary allylic hydroperoxide (**15**) in the presence of molecular oxygen, as established experimentally in Ref. 39.

[†] The rather unexpected spontaneity of this apparently complex transformation shown in Scheme 1 has been demonstrated to be the result of the proximity of the 12-carboxylic acid group[42] to the $\Delta^{4,5}$-double bond in **2**, which assisted/catalysed both of the autoxidation reactions and the Hock cleavage reaction of the tertiary allylic hydroperoxide intermediate **15**.

[‡] For all compounds with the suffix 'a' in this paper, the [15-CH_3] group has been replaced by [15-$^{13}C^{2}H_3$]; for compounds labelled with the suffix 'b', the [15-CH_3] group has been replaced by [15-$C^{2}H_3$]; for compounds labelled with the suffix 'c', the [15-CH_3] group has been replaced by [15-$^{13}CH_3$]. The suffix '*' indicates an unspecified isotopic enrichment at the 15-position.

of **2a**, or as intact plants, which had been washed clean of soil and immersed by the root, was assayed by immediately extracting these plants with CH_2Cl_2, following the complete uptake of the feeding solution. (The feeding solution was taken up by the plants over a period of a few hours, when administered at a level of roughly 1 ml (containing ca. 2 mg of **2a**) to every 5 g of fresh plant material.)

The resulting crude extracts were analysed directly by ^2H NMR spectroscopy which consistently demonstrated that both intact (root-fed) and 'cut' (stem-fed) *A. annua* plants assimilated between 50–80% of the labelled dihydroartemisinic acid (**2a**) under these conditions. A single doublet peak (δ_D 1.63 ppm, $^1J_{CD}$=19.1 Hz), corresponding to the labelled dihydroartemisinic acid precursor, was observed in all the ^2H NMR spectra of the CH_2Cl_2 extracts which were made in this preliminary study, indicating that no discernible metabolism had occurred over the relatively short period of time used for feeding. By extracting root compartments, stem compartments and leaf/flower compartments separately, it was then possible to estimate the distribution of this labelled precursor within the plant. For plants fed via the cut stem, it was found that there was more or less equal recovery of label between the leaf/flower compartments and the stem compartment (25–40% of the labelled precursor was recovered from each). A parallel distribution was observed between the various aerial parts of the intact plants, with 5–20% remaining in the root. The evident similarities between the two feeding methods ('cut' plants fed via the stem and intact plants fed via the root) appeared to demonstrate that the root of *A. anuua* offered no physiological barrier to the uptake of dihydroartemisinic acid and that the labelled precursor could then be efficiently redistributed throughout the various plant tissues, following its uptake by the root. This was a remarkable result, as it suggested that the most 'natural' feeding method which we could devise ('natural' in the sense that this technique involves the minimum possible disturbance to, and manipulation of, the biological system; and is, therefore, more likely to produce results which most closely mimic the metabolic fate for dihydroartemisinic acid that is naturally present in *A. annua* plants) also resulted in an exceptionally high incorporation of the labelled dihydrortemisinic acid precursor into the tissues of *A. annua* (up to 80% recovery). In consequence, all the feeding experiments which are described in Sections 2.2–2.4 were performed using intact *A. annua* plants which were fed with one of the labelled precursors **2a**, **2b** or **2c** via the root.

2.2. Time-course study of the in vivo transformations of [15-$^{13}C^2H_3$]-dihydroartemisinic acid (2a) which was fed to intact *A. annua* plants via the root

The experimental design for the study of the metabolism of dihydroartemisinic acid in *A. annua* plants which is described in this section involved feeding 21 intact plants with 5 mg each of compound **2a** via the root, using the optimized procedure which was developed in Section 2.1. The plants were kept alive hydroponically by periodic replacement of water in their feeding vials, and CH_2Cl_2 extracts were made at approximately daily intervals until the experiment had been completed after six days. Each extraction event involved groups of three plants which were separated into a root compartment and 'aerial parts',[§] then extracted individually (triplicate replication) by dichloromethane and analysed immediately by ^2H NMR spectroscopy, as described in Section 2.1. ^2H NMR spectroscopic analysis of the crude extracts of the aerial parts made at 10, 24, 33, 48, 72, 96 and 144 h after the commencement of feeding indicated substantial changes, as is shown in Figure 2 (in which one representative ^2H NMR spectrum is shown from each of the triplicate extracts made at these times after feeding **2a**). In these spectra, the doublet at δ_D 1.63 ppm, ($^1J_{CD}$=19.1 Hz), corresponding to the [15-$^{13}C^2H_3$] label in compound **2a**, can clearly be seen to decline in abundance over the course of the experiment relative to the other peaks in the ^2H NMR spectrum and to be replaced, most obviously, by a doublet at δ_D 1.68 ppm. Inspite of the complexity of these spectra, one can also appreciate the appearance of several other doublets, in particular at δ_D 1.27, 1.34 and 2.14 ppm.

Although the largish one-bond $^{13}C-^2H$ coupling constant ($^1J_{CD}$=19.1 Hz) of all the metabolites which were doubly-labelled at the 15-position made a detailed visual analysis of the one-dimensional ^2H NMR spectra from the crude extracts of the aerial parts difficult, it did allow for the direct analysis of these crude extracts by two-dimensional $^{13}C-^2H$ correlation NMR spectroscopy.[44] $^{13}C-^2H$ COSY[44,45] is an unusual NMR technique which has apparently found little application in the literature, perhaps due both to the need for special hardware to provide ^2H excitation and decoupling (although modern NMR spectrometers do now routinely incorporate the necessary hardware) and the requirement for doubly-labelled sample molecules. It is, however, an excellent tool for the analysis of the crude plant extracts which were obtained in this experiment, since it allows the ^{13}C chemical shift at the 15-position of metabolites to be also established, for ^{13}C nuclei which are connected by a single bond to ^2H nuclei at the same position—thereby enabling metabolites to be identified with greater confidence from characteristic chemical shifts in both the ^{13}C and ^2H dimensions (see Ref. 45 for a detailed discussion of the preliminary application of this technique to a biosynthetic experiment). $^{13}C-^2H$ COSY analysis of the crude plant extracts indicated that the most abundant deuterium resonances at δ_D 1.68, 1.27, 1.34 and 2.14 ppm were connected by a single bond to carbon resonances at δ_C 22.6, 23.6, 22.2 and 29.1 ppm, respectively (a two-dimensional peak was also observed in most samples at δ_D 1.63/δ_C 22.9 ppm, corresponding to the labelled precursor **2a**).

When allowance was made for the effects of ^2H isotopic enrichment on ^{13}C chemical shift (resulting in a ca. 0.3 ppm upfield shift in the position of ^{13}C resonance for each directly attached deuterium atom),[43,44] the isotopically-normal values for each of these four metabolites could then

[§] Since preliminary experiments (not discussed) had indicated that there was little difference between the leaf/flower compartment and the stem compartment, as regards the incorporation and subsequent transformations of labelled **2**, these two compartments were combined together and treated as the 'aerial parts' in this experiment.

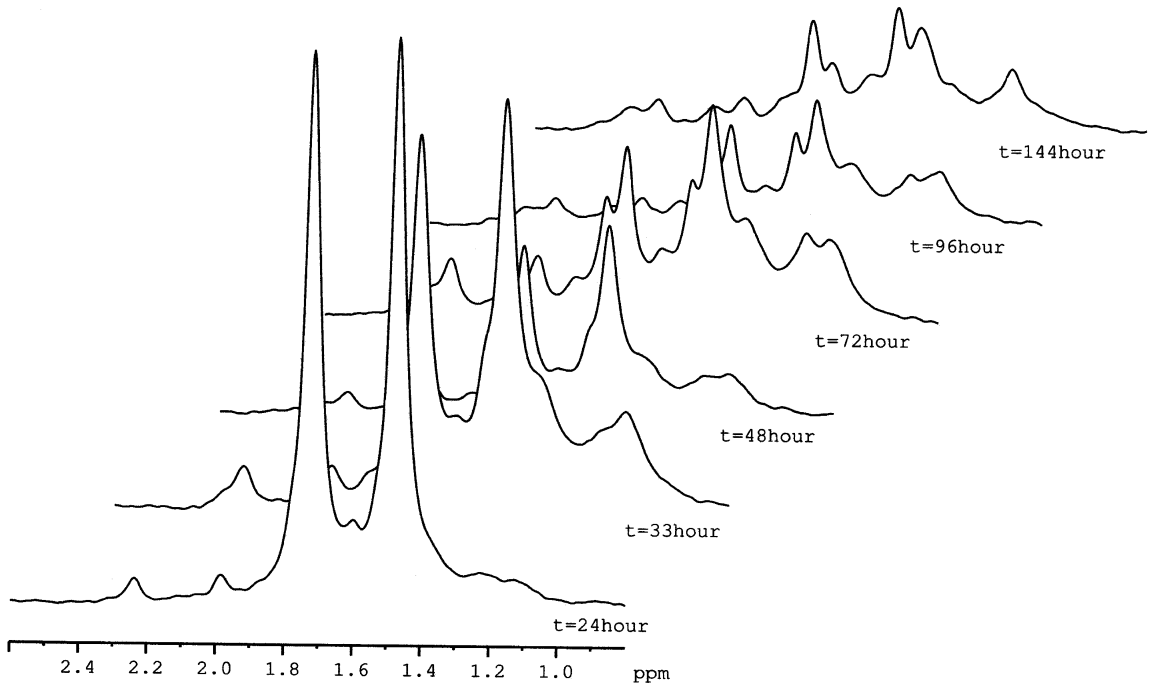


Figure 2. Expansion of the aliphatic region for representative ^{2}H NMR spectra from the aerial parts of *A. annua* plants which were fed with compound **2a** and then extracted in triplicate by CH_2Cl_2 after 24, 33, 48, 72, 96 and 144 h.

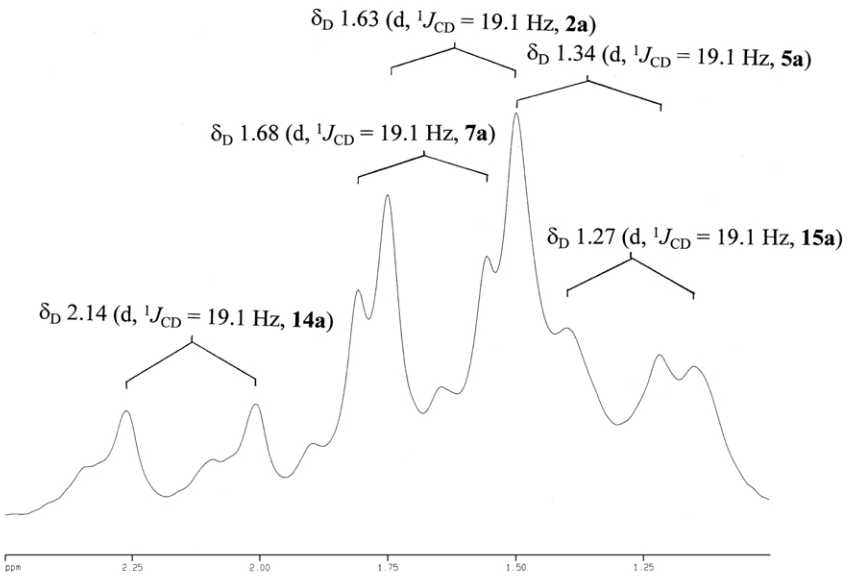
be inferred as δ_C 23.5, 24.5, 23.1 and 30.0 ppm, respectively, assuming that there has been complete retention of the ^{2}H label in the 15-methyl group (note that the 15-position of isotopically-normal dihydroartemisinic acid resonates at δ_C 23.8 ppm which is in good agreement with the carbon chemical shift observed at δ_C 22.9 ppm for labelled **2a** in this $^{13}C-^{2}H$ COSY experiment, according to such an analysis). The deuterium and carbon chemical shifts at the 15-position for the four metabolites which are most clearly resolved in Figure 2 would then be entirely consistent with their assignments as compounds **7a**,[2,43,45] **15a**,[2,45] **5a**[2,45] and **14a**[39,45] respectively, based on the fully-assigned NMR data which has been reported in the literature for each of these compounds. The ^{13}C and ^{1}H chemical shifts at the at the 15-position for the various secondary metabolites that have subsequently been isolated in this study (which constitute a large percentage of all of the 11,13-dihydro-amorphane/cadinane sesquiterpenes known from *A. annua*) are presented in Table 1 in order to illustrate that these two chemical shift parameters can indeed be used with some confidence in assigning the structures of compounds **5**, **7**, **14** and **15** (references are given to the fully assigned ^{13}C and ^{1}H NMR data, as originally reported, in each case).

Returning to the analysis of the one-dimensional ^{2}H NMR spectra, such as those shown in Figure 2, we next attempted to estimate the relative percentage amount for each of the labelled compounds **2a**, **5a**, **7a**, **14a** and **15a** that was present in all of the 21 crude extracts of the aerial parts of *A. annua* which had been fed with **2a**. In the event, computational simulation of each spectrum proved to be the only realistic way to achieve a semi-quantitative analysis: each deuteriated component of the extract was modelled as a doublet of Lorentzian lines ($^{1}J_{CD}$=19.1 Hz; line width at half height approximately 2.5–5.5 Hz) centred at δ_D 1.63, 1.34, 1.68, 2.14 and 1.27 ppm, respectively. Allowing for the presence of a large number of additional minor metabolites in these spectra, it can be seen from the examples given in Figure 3 that a good correspondence could be achieved between the ^{2}H NMR spectra and their simulations using this approach (several minor doublet peaks at δ_D 2.19, 1.78, 1.51 and 1.44 were also included in

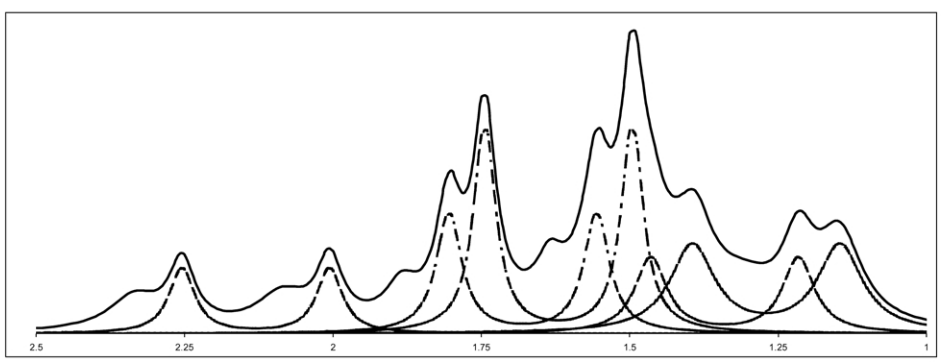
Table 1. ^{1}H and ^{13}C chemical shifts at the 15-position for all of the metabolites **1**, **2**, **5**, **7**, **11** and **14**–**25** which have been reported in the literature.

11,13-Dihydro amorphane/cadinane sesquiterpenes from *A. annua*	δ_H	δ_C	Ref.	11,13-Dihydro amorphane/cadinane sesquiterpenes from *A. annua*	δ_H	δ_C	Ref.
1	1.44	25.2	47	**17**	1.36	23.6	6
2	1.63	23.8	2	**18**	1.53	23.9	48
5	1.35	23.1	2,45	**19**	1.78	21.2	2, 5
7	1.68	23.5	2,43,45	**20**	4.93, 4.91	114.5	2, 5
11	1.39	26.6	2,5	**21**	4.91, 4.84	106.6	2, 49
14	2.14	30.0	6,39,45	**22**	5.05, 4.81	105.1	2
15	1.29	24.5	2,45	**23**	1.78	18.8	2
16	1.70	24.1	4	**24**	1.31	23.6	6,43
				25	1.33	24.2	5

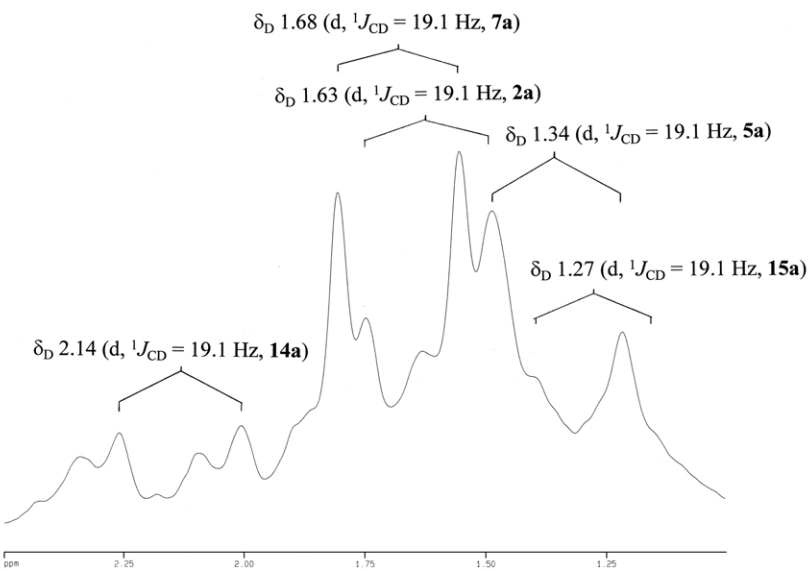
(a)


δD 1.63 (d, 1JCD = 19.1 Hz, 2a)
δD 1.34 (d, 1JCD = 19.1 Hz, 5a)
δD 1.68 (d, 1JCD = 19.1 Hz, 7a)
δD 1.27 (d, 1JCD = 19.1 Hz, 15a)
δD 2.14 (d, 1JCD = 19.1 Hz, 14a)
ppm
2.25
2.00
1.75
1.50
1.25

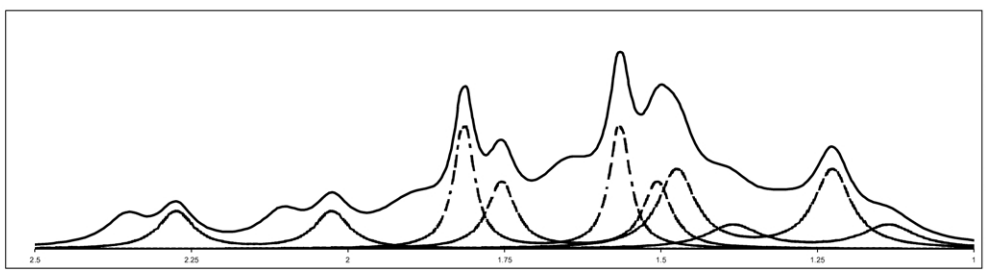
(b)


2.5
2.25
2
1.75
1.5
1.25
1

(c)


δD 1.68 (d, 1JCD = 19.1 Hz, 7a)
δD 1.63 (d, 1JCD = 19.1 Hz, 2a)
δD 1.34 (d, 1JCD = 19.1 Hz, 5a)
δD 1.27 (d, 1JCD = 19.1 Hz, 15a)
δD 2.14 (d, 1JCD = 19.1 Hz, 14a)
ppm
2.25
2.00
1.75
1.50
1.25

(d)


2.5
2.25
2
1.75
1.5
1.25
1

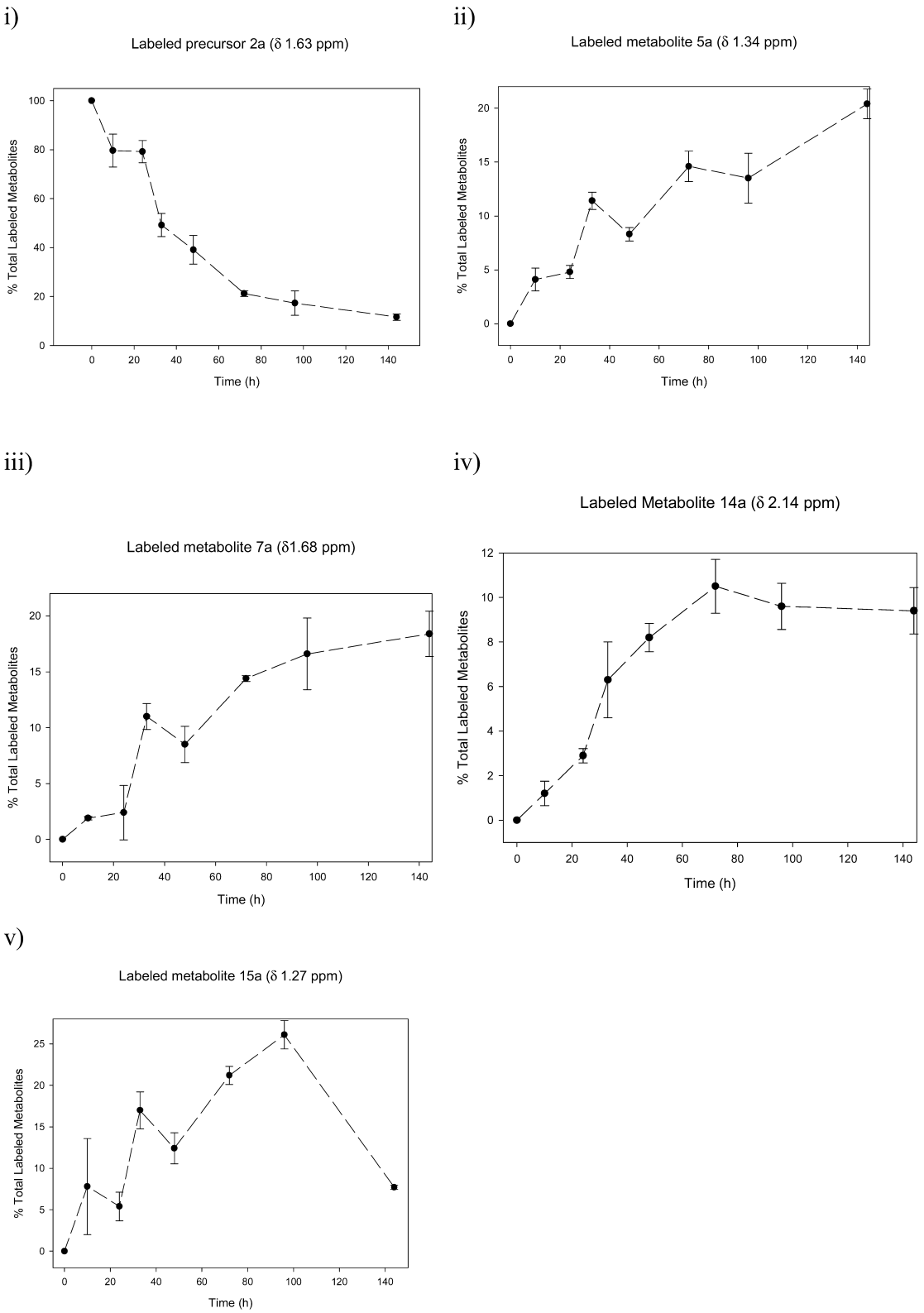
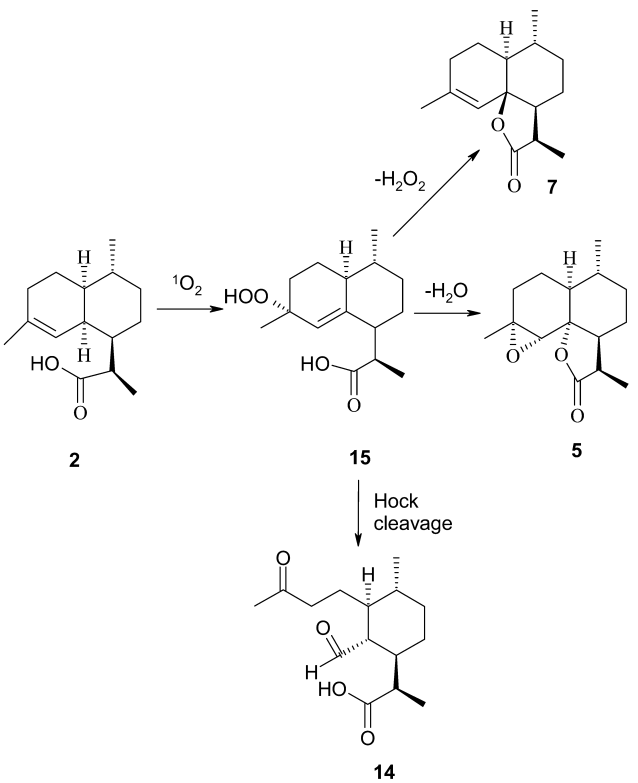


Figure 4. Variation with time in the percentage amounts of (i) **2a**, (ii) **5a**, (iii) **7a**, (iv) **14a** and (v) **15a** in the crude aerial extracts of *A. annua* plants fed with **2a**. Each data point is the average of the percentage metabolite amount from three replicates, each of which was estimated by computational simulation (Fig. 3). The corresponding standard deviations are indicated by error bars.

Figure 3. Two examples of the ^{2}H NMR spectra of the extracts of the aerial parts made at (a) 72 h and (c) 144 h after feeding *A. annua* with **2a**; and the corresponding computer simulations (b) and (d) which were used to estimate the percentage amounts of compounds **14a**, **7a**, **2a**, **5a** and **15a** (in order of decreasing deuterium chemical shift at the 15-position) in these crude extracts (the contribution of the doublets from the major components of the extract to the overall spectral simulation are shown explicitly by dotted lines).

these modeling simulations in order to obtain accurate fits to experimental data, but are not explicitly shown as 'dotted' lines in Figure 3 for overall ease of visualization—the origins of these minor peaks are discussed in the next section).

We then proceeded to make an approximate quantification of the extent of the in vivo transformations of **2a** with time, based on such simulations. The result is shown in Figure 4 as a plot of the estimated percentage amounts of each of compounds **2a**, **5a**, **7a**, **14a** and **15a** against time. This figure confirms a general trend in which the amount of the labelled precursor **2a** has declined with time at approximately the same rate at which all of metabolites **5a**, **7a** and **14a** have increased. However, the behavior of metabolite **15a**, which increased at the start of the experiment, reached a maximum after around 96 h, and then declined in abundance was more consistent with its role as an intermediate[46] in the conversion of **2a** to each of **5a**, **7a** and **14a**. Our conclusion from this analysis was therefore, that the most dominant pathway for the transformation of dihydroartemisinic acid in the aerial parts of *A. annua* is: **2→15→(5+7+14)** as is shown in Scheme 2. A similar analysis of the root extracts (not shown) suggested a comparable metabolic profile to that described for the aerial parts, but with reactions occurring at a very much slower rate (ca. 80% of the labelled precursor remained unchanged at the end of the experiment).



Scheme 2. The most dominant pathways for the transformation of dihydroartemisinic acid (**2**) in vivo, as established by 1D and 2D NMR studies of crude *A. annua* plant extracts (Figs. 2–4 and 6–8). Note that these pathways were all also observed from in vitro studies of the spontaneous autoxidation of **2** (Ref. 39).

2.3. Time-course study of the in vivo transformations of [15-C^2H_3]-dihydroartemisinic acid (2b) in both living and dead *A. annua* plants which were fed by the root

In a second experiment, 45 *A. annua* plants were individually fed via the root with 4.4 mg each of [15-C^2H_3]-dihydroartemisinic acid (**2b**). Three plants were extracted in the morning, soon after feeding had been completed, and these extracts, for which no metabolism was observed, were designated as the 'zero-time' point (0 h). The remaining plants were divided into two groups of 21 plants each: one group was kept alive hydroponically, as previously, and the second group was deprived of water and allowed to die naturally by desiccation. Leaf/flower, stem and root compartments for each plant were extracted separately at intervals over a four-day period (employing triplicate replication as before). As might be expected, the weight of the group of plants which was deprived of water after feeding (expressed as a percentage of the total weight of the plant at the time of feeding) decreased drastically over the course of the experiment (Fig. 5) and we believe that these plants were dead roughly one day after commencement of the extraction regime (or, at least, that the intracellular environment had become sufficiently dehydrated that enzymatic processes could no longer occur, which is equivalent for the purposes of the discussion which follows). By contrast, the extraction weight of the 'live' group of plants remained relatively constant (at around 60–70% of the initial weight—data not shown) and these plants were visually quite healthy over the entire course of the experiment.

Extracts were made from both live and dead groups of plants on an evening/morning schedule at 8, 24, 32, 48, 56, 72 and 80 h after the zero-time point and were immediately analysed by ^2H NMR spectroscopy. The absence of $^{13}C-^2H$ coupling at the 15-position of **2b** and its metabolites made interpretation of the one-dimensional ^2H NMR spectra from this experiment much simpler than was the case when feeding compound **2a** in Section 2.2 (Fig. 2). The most dominant peaks seen in these spectra for both the 'live' and 'dead' groups of plants appeared as singlets at the same chemical shifts as previously (δ_D 1.68, 1.27, 1.34 and 2.14 ppm). In addition, several minor singlet peaks were now clearly resolved at δ_D 2.20, 1.78, 1.51 and 1.44 ppm, becoming more obvious for the later extracts in particular (Figure 6—only examples from the 'dead' group of plants are shown, but the 'live' group gave very similar results).

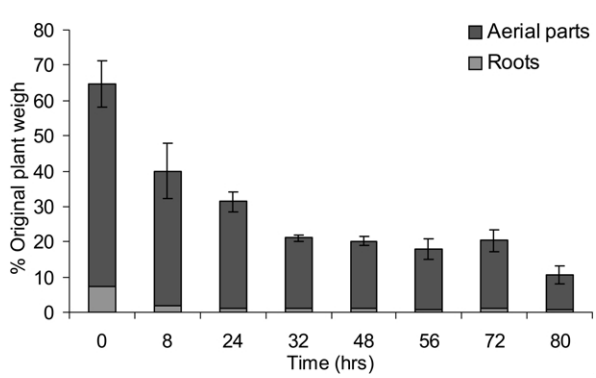


Figure 5. Weight at extraction of *A. annua* plants which were allowed to die by desiccation after feeding of compound **2b** via the root, expressed as a percentage of their weight at the time of feeding (each entry is an average of three replicates).

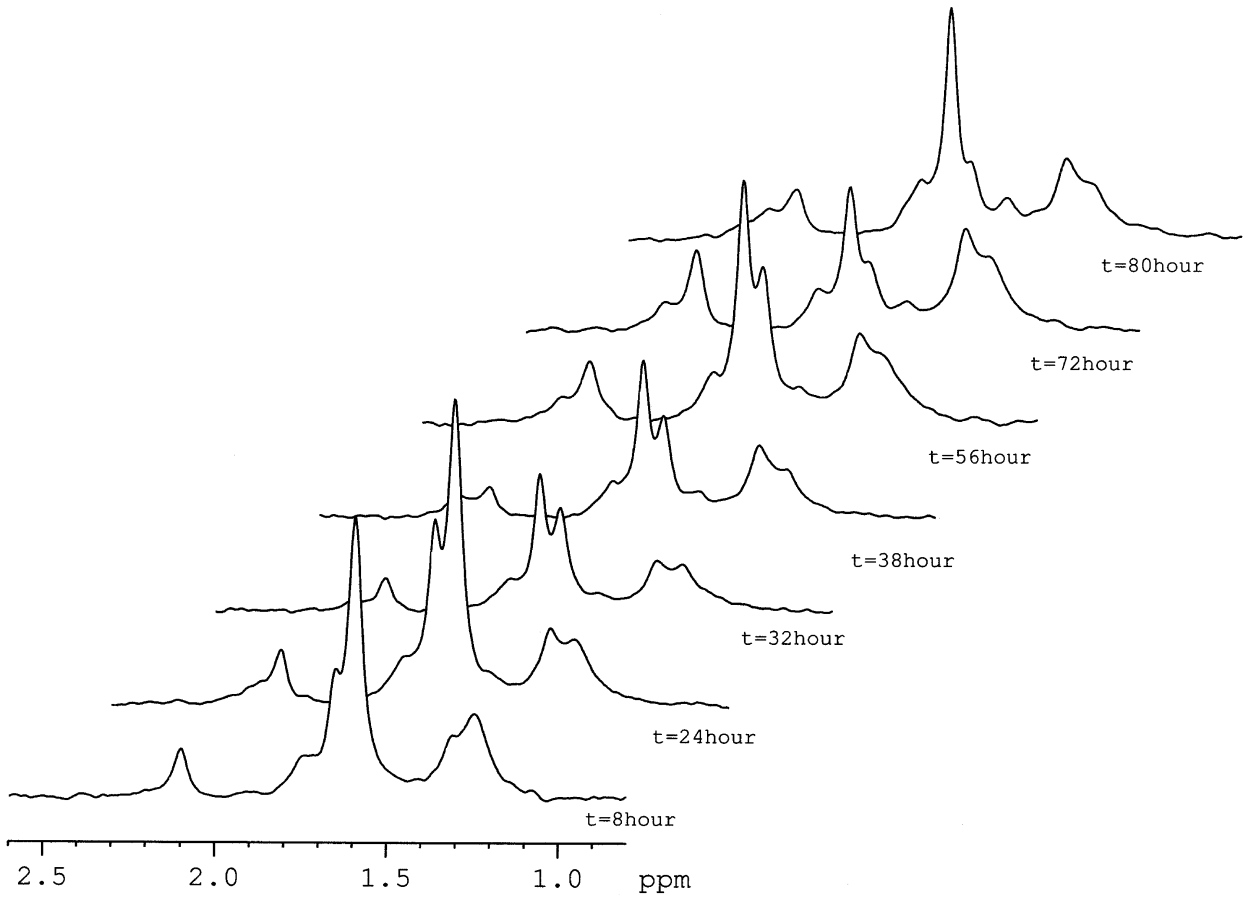


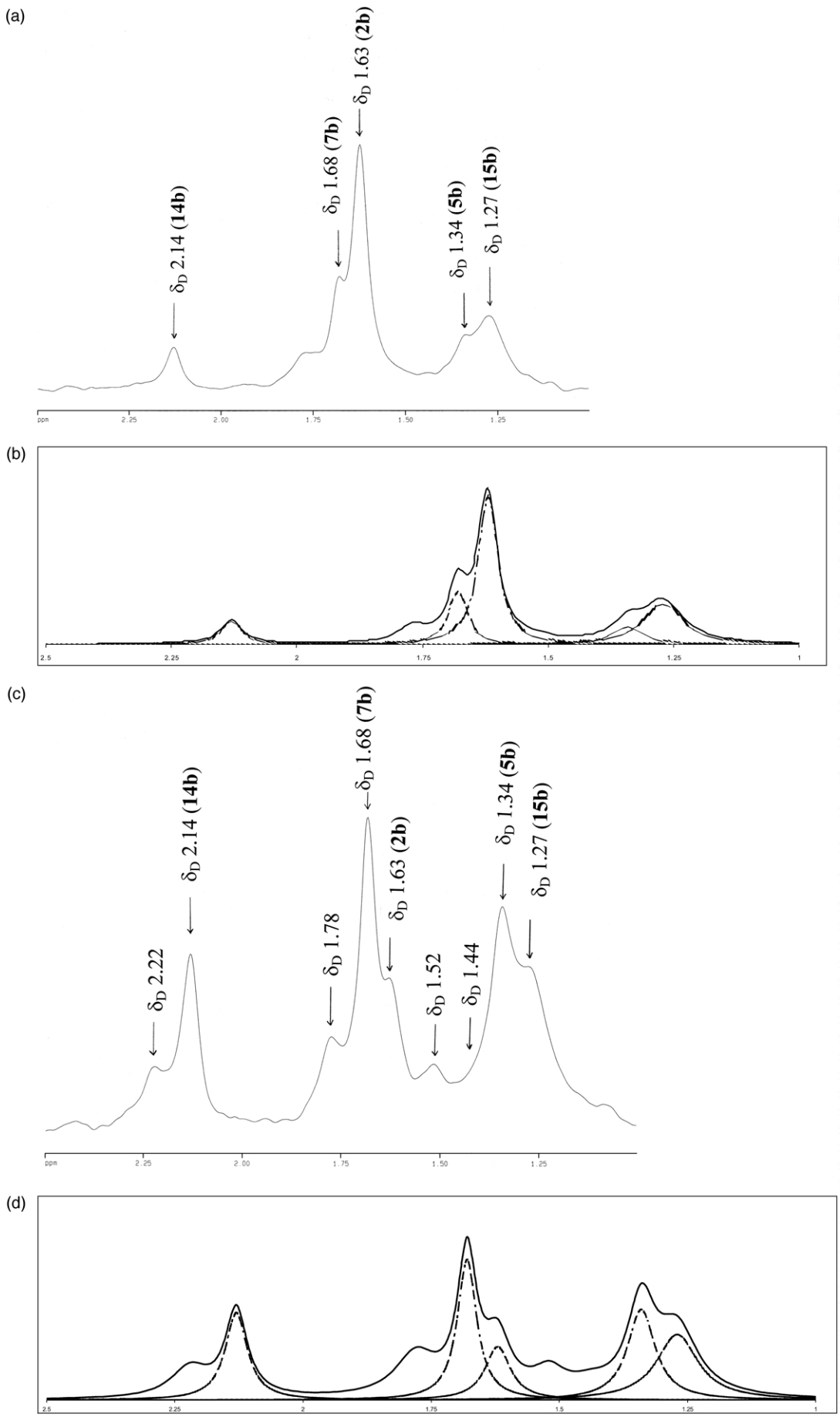
Figure 6. Expansion of the aliphatic region for representative ^{2}H NMR spectra from the aerial parts of *A. annua* which were allowed to die by desiccation after feeding with **2b** and then extracted in triplicate by CH_2Cl_2 after 8, 24, 32, 48, 56, 72 and 80 h.

Furthermore, a broad poorly resolved resonance was also noted in the alkene region of the spectrum between δ_D 4.7 and 5.1 ppm (not shown in Figure 6) in several of the spectra taken towards the end of the experiment.

Based on the results of the previous section, the more intense deuterium resonances at δ_D 1.63, 1.68, 1.34, 1.27 and 2.14 ppm were proposed to correspond to the labelled [15-C^{2}H$_3$] group in compounds **2b**, **7b**, **5b**, **15b** and **14b** respectively, and the percentage amounts of each of these compounds in all of the extracts made in this experiment could then be estimated reasonably accurately by computational simulation (fitting a single Lorentzian line at each chemical shift), as is shown in Figure 7.

The variation over time in the percentage amounts of the precursor **2b** and each of the four most abundant metabolites, for the group of plants which was allowed to die by desiccation immediately after feeding of **2b**, is depicted in Figure 8. These results were almost identical with those obtained for the 'live' group of plants in this same experiment (results not shown) and also with the results for plants which had been kept alive in the previous section, as summarized in Figure 4. In particular, compound **15b** again appeared to be an intermediate, which reached its peak at around 32 h. It is worth noting that the rates of decline in the precursor and its transformation into products **5b**, **7b** and **14b** in this second experiment (Fig. 8) were also somewhat faster than for the first (Fig. 4), which may be the result of a warmer average temperature and continuous sunshine during the hours of daylight (see Section 4.1.4). One can perhaps discern a diurnal fluctuation in the amounts of the precursor **2b**, which tends to be more abundant in those extracts made in the morning (24, 48 and 72 h) and to have undergone an increased rate of conversion to its metabolites **5b**, **7b** and **14b** in the extracts which were made in the late afternoon (8, 32, 56 and 80 h), superimposed on the general trend. This would be consistent with a continuous replenishment from the root of **2** in the aerial parts during both day and night time and with an increased rate of autoxidation of **2** during the hours of daylight. We therefore, suggest that **2b** undergoes chemical transformations in the 'dead' group of plants in exactly the same way as for the 'live' group (i.e. via the pathway **2b**→**15b**→(**5b**+**7b**+**14b**)). Analysis of the root extracts from both the live and dead groups of plants showed only very limited transformations of **2b**, as had been observed previously for the precursor **2a** in Section 2.2.

Finally, we turn to the minor peaks which were observed in some of the ^{2}H NMR spectra from this experiment at δ_D 2.19, 1.78, 1.51 and 1.44 ppm and which were most clearly resolved for the later extracts in Figure 6 (there was also a broad poorly-resolved resonance at δ_D 1.30–1.40 ppm in some of these spectra). In order to determine the identities of the minor metabolites of **2b** which were responsible for these peaks, we combined together all the crude plant extracts from this experiment and subjected them to


(a)
δD 2.14 (14b)
δD 1.68 (7b)
δD 1.63 (2b)
δD 1.34 (5b)
δD 1.27 (15b)
ppm
2.25
2.00
1.75
1.50
1.25
(b)
2.5
2.25
2
1.75
1.5
1.25
1
(c)
δD 2.22
δD 2.14 (14b)
δD 1.78
δD 1.68 (7b)
δD 1.63 (2b)
δD 1.52
δD 1.44
δD 1.34 (5b)
δD 1.27 (15b)
ppm
2.25
2.00
1.75
1.50
1.25
(d)
2.5
2.25
2
1.75
1.5
1.25
1

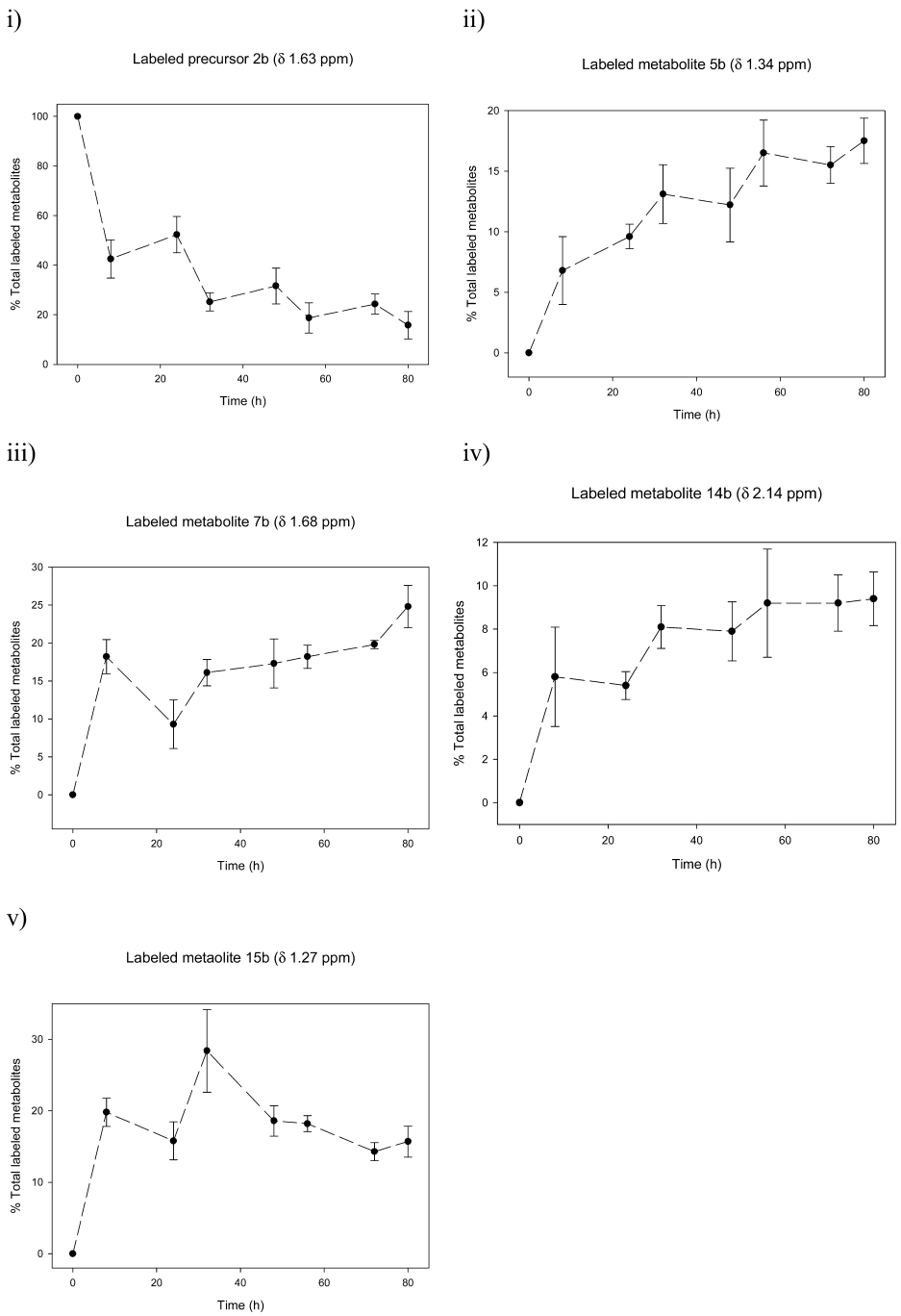
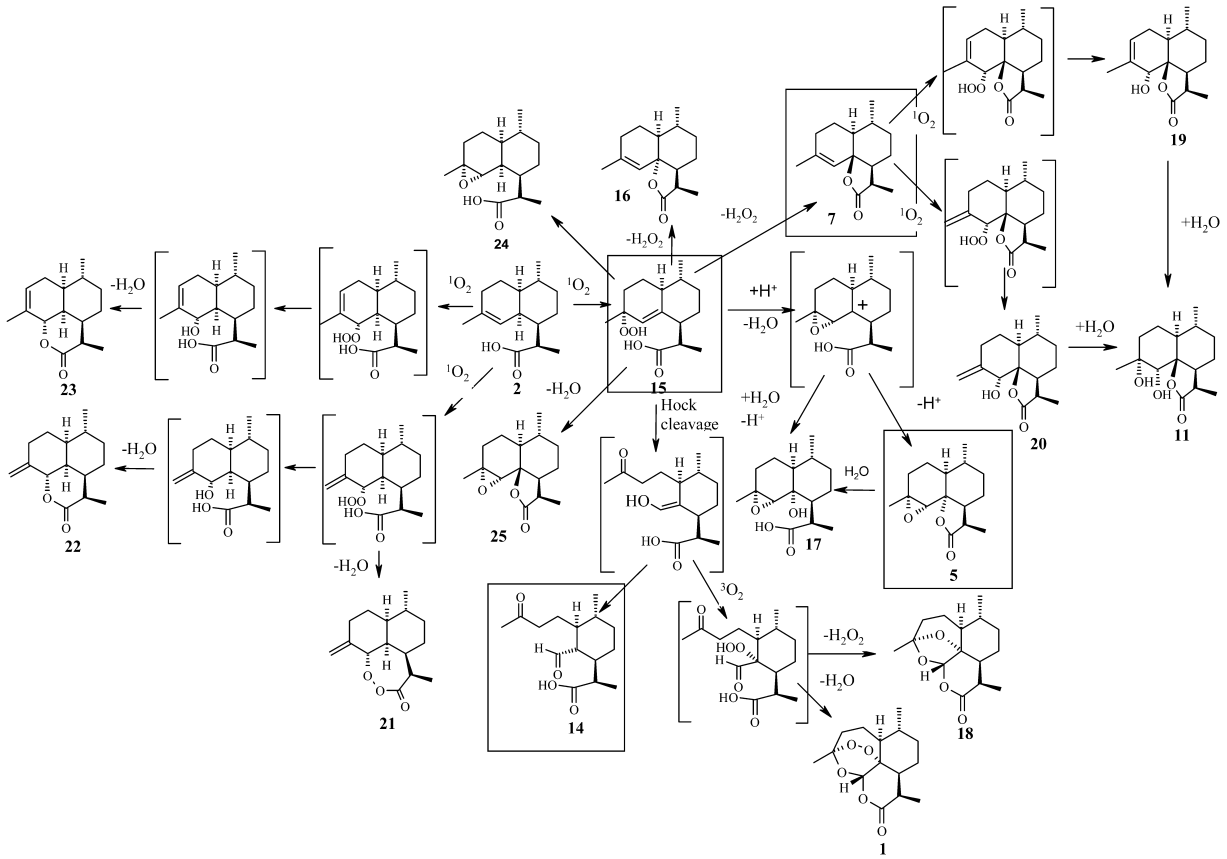


Figure 8. Variation in the percentage amounts of (i) **2b**, (ii) **5b**, (iii) **7b**, (iv) **14b** and (v) **15b** with time in the crude aerial extracts of *A. annua* plants which were fed with **2b** and allowed to die by desiccation. Each point is the average of the percentage amount from three replicates, each of which was estimated by computational simulation (Fig. 7). The corresponding standard variations are indicated by an error bar.

chromatographic separation, as is described in the next section.

2.3.1. Chromatographic separation of the combined extracts of the aerial compartment of *A. annua* plants which had been fed with 2b. Extensive HPLC separations of the combined crude extracts obtained from the feeding of [15-C^2H_3]-dihydroartemisinic acid (**2b**) to both the 'live' and 'dead' groups of plants described in the previous section resulted in the isolation of 16 deuterium-labelled 12-carboxy-11,13-dihydro-amorphane and cadinane sesquiterpenes (Scheme 3 and Table 2) As expected, compounds **5b**,

Figure 7. Two examples of the ^2H NMR spectra of extracts of the aerial parts of *A. annua* made at (a) 8 and (c) 72 h after feeding with **2b** (the plants were allowed to die by desiccation after feeding); and (b) and (d) the corresponding computer simulations which were used to estimate the percentage amounts of compounds **14b**, **7b**, **2b**, **5b** and **15b** (in order of decreasing chemical shift at the 15-position) in these crude extracts (the contribution of the singlets—due to the major components of the extract—to the overall spectral simulation are shown by dotted lines; contributions from minor components are not explicitly shown for overall ease of visualization).



Scheme 3. Proposed routes for the in vivo transformations of dihydroartemisinic acid (**2**) (mechanisms are based largely on previous in vitro studies) leading to the 16 amorphane and cadinane metabolites which were isolated in feeding experiments with *A. annua* in Section 2.3. Boxed structures represent the most abundant metabolites to be isolated (as shown in Scheme 2).

7b and **14b**, which are the products of the most dominant pathways shown in Scheme 2, were obtained in large quantities. The amounts of all 16 metabolites obtained by chromatographic separation are indicated in Table 2, together with the ratio of the [15-C^2H_3]/[15-CH_3] isotopomers for each metabolite, as determined by ^1H, ^{13}C NMR and mass spectrometry. (Note that since several HPLC purification steps were involved in obtaining each metabolite and, because

Table 2. Amounts and isotopic composition, as determined by various spectroscopic techniques, of [15-C^2H_3]-labelled metabolites which were isolated chromatographically after feeding **2b** to *A. annua* plants.

Compound (δ_D (ppm) at the 15-position)[a]	Amount isolated (mg)	Percentage of the [15-C^2H_3]-isotopomer as a total of both the labelled [15-C^2H_3]- and unlabelled [15-CH_3]-isotopomers recovered from the plant extracts, as estimated by three spectroscopic techniques (%)		
		From ^1H NMR	From ^{13}C NMR[b]	From MS
1b (1.44)	9.0	<5	<5	7
2b (1.63)	1.0	>40	>90	83
5b (1.35)	0.6	ca. 70	ca. *70*	—
7b (1.68)	3.8	>70	ca. 60	75
11b (1.39)	0.1	>50	ca. 50	70
14b (2.13)	0.3	ca. 70	ca. 70	75
15b (1.29)	0.8	>70	75	—
16b (1.68)	0.1	>70	>80	83
17b (1.35)	0.3	>70	ca. 70	74
18b (1.52)	1.5	>50	ca. 70	63
19b (1.78)	0.1	>60	—	73
20b (4.94 (br))	0.1	67	>80	73
21b (4.93, 4.85)	0.3	16	<5	17
22b (5.07, 4.86)	1.1	73	ca. 80	75
23b (1.78)	0.1	>50	—	—
24b (1.31)	0.1	>70	—	77
25b (1.32)	0.1	>70	>80	88

[a] This deuterium chemical shift was used for identifying metabolites in the crude plant extracts.
[b] Feeding experiments with [15-$^{13}CH_3$] labelled dihydroartemisinic acid (**2c**) in Section 2.4, generally confirmed the results shown in this column.

the recoveries at each step were unknown and likely to be quite variable, the amounts shown in Table 2 should be treated as underestimates and as only being semi-quantitative). Although a large number of other natural products were, of course, also isolated from this chromatographic separation (see Ref. 6 for an indication of the non-sesquiterpenoid metabolites that are typically also found in *A. annua*), none contained any detectable amount of ^{2}H label.

Of the 16 labelled metabolites of **2**, 15 have already been reported as natural products from *A. annua*, including:¶ artemisinin (**1**),[47] dihydroarteannuin B (**5**),[2,45] dihydro-*epi*-deoxyarteannuin B (**7**),[2,45] arteannuin M (**11**),[2,5] the keto-aldehyde **14**,[6,39] the tertiary hydroperoxide of dihydroartemisinic acid (**15**),[2,45] dihydro-deoxyarteannuin B (**16**),[4] the β-epoxy alcohol **17**,[6] deoxyartemisinin (**18**),[48] arteannuin K (**19**),[2,5] arteannuin L (**20**),[2,5] arteannuin H (**21**),[2,49] arteannuin I (**22**),[2] arteannuin J (**23**)[2] and α-epoxy-dihydroartemisinic acid (**24**).[6,43] The only metabolite not to have been obtained previously as a natural product was dihydro-*epi*-arteannuin B (**25**), although this compound has recently been reported as an intermediate in the chemical synthesis of the natural product, arteannuin O (**12**).[5] Based on this chromatographic separation, it can be concluded that the minor peaks seen in the ^{2}H NMR spectra in Figures 6 and 7 at δ_D 1.78, 1.51 and 1.44 ppm were probably due to arteannuins K and J (**19b/23b**), deoxyartemisinin (**18b**) and artemisinin (**1b**), respectively. The poorly defined resonances between 4.7 and 5.1 ppm which were observed in some of the crude extracts might have been due to some or all of arteannuin L (**20b**), arteannuin H (**21b**) and arteannuin I (**22b**). The broad 'hump' of poorly resolved resonances in the range δ_D 1.30–1.40 ppm, commonly encountered in the later extracts, were perhaps due to some or all of the metabolites **11b**, **17b**, **24b** and **25b**. Deuterium signals were not directly observed in the ^{2}H NMR spectra of the crude plant extracts from dihydro-deoxyarteannuin B (**16b**) as the weak resonance for the 15-position in this compound (δ_D 1.68 ppm) would have been completely obscured by the much stronger peaks from the principal metabolite **7b** (also at δ_D 1.68 ppm). The identity of the peak at δ_D 2.19 ppm, which was quite significant in many crude ^{2}H NMR spectra (particularly the later ones), remains uncertain, as no metabolite exhibiting this deuterium resonance was obtained after chromatographic separation. However, we suspect that it may be the 5-carboxylic acid analogue of the natural product **14b**; as the aldehyde group in this compound is known to undergo facile spontaneous oxidation[39] to yield a dicarboxylic acid product which may have been too polar to be isolated by the normal phase chromatographic separation procedures employed in this study.

A careful analysis of the ^{1}H NMR, ^{13}C NMR and mass spectra for all of the metabolites from the chromatographic separation (Table 2) indicated that the [15-C^{2}H$_3$]-isotopomer (i.e. '**b**' suffix) accounted for roughly 70% of the total metabolite recovered (comprising both the labelled and the isotopically-normal [15-CH$_3$] form), with only two exceptions. These were artemisinin (**1**) and arteannuin H (**21**), both of which were isolated as a mixture of approximately 5–15% of the deuterium-labelled isotopomer with the unlabelled (isotopically-normal) form. In no case was there any evidence whatsoever for the presence of 'partially' labelled forms of any of these metabolites, which would have been easily detectable by the presence of molecular ions for the [15-C^{2}H$_2$H]- and [15-C^{2}HH$_2$]-isotopomers in mass spectrometry. Since there was therefore, no reason to propose the existence of metabolic processes which would cause the deuterium label to be progressively 'lost' from the 15-position in vivo, we believe that the isotopically-normal forms of all of these metabolites must represent *bona fide* natural products, which would have been present in *A. annua* in any case, even if labelled dihydroartemisinic acid had not been administered to the plants. The percentage ratio of the [15-C^{2}H$_3$]-isotopomer to the [15-CH$_3$]-isotopomer should therefore, give an indication of the extent, relative to the unperturbed system, by which artificially administered **2b** is being converted into the various secondary metabolites that are typical of *A. annua*, when fed to the plant via the root. If this is so, then the conclusion must be that when dihydroartemisinic acid was fed to *A. annua* plants via the root, two of the 16 metabolites into which it was converted, compounds **1** and **21**, were accumulated to a significantly lesser extent than all of the other fourteen (**5b**, **7b**, **11b**, **14b**–**20b** and **22b**–**25b**), as compared with the 'natural' situation.

2.4. Time-course study of the transformations of [15-^{13}CH$_3$]-dihydroartemisinic acid (2c) which was fed to *A. annua* plants via the root

Several experiments were also performed involving the feeding of [15-^{13}CH$_3$]-dihydroartemisinic acid (**2c**) to *A. annua* plants via the root. However, these experiments were, in general, found to be much more difficult to analyse than for the feeding of either **2a** or **2b**, as the option for an initial screening of crude plant extracts for labelled compounds by ^{2}H NMR spectroscopy, which in Sections 2.2 and 2.3 had clearly and rapidly indicated the presence of—and transformations undergone by—the ^{2}H label without any interference from the (much more abundant) non-labelled components of the plant matrix, no longer existed. The crude extracts of plants which had been fed with compound **2c** always had to be subjected first to chromatographic separation in order to establish the presence of a ^{13}C-isotopically-enriched metabolite, and this was a tedious and time-consuming task without the benefit of the ^{2}H label, which had enabled the most promising chromatographic fractions to be rapidly targeted in Section 2.3.1. However, those ^{13}C-labelled metabolites which were chromatographically isolated from these experiments fully confirmed the results established from feeding experiments with both **2a** and **2b** in the previous sections. In particular, the intensity of the ^{13}C resonance at the 15-position, relative to other ^{13}C resonances in each of the purified metabolites could be used to estimate the percentages of the 15-[^{13}CH$_3$]-labelled forms of metabolites which were derived from **2c** vs. the 'natural' level of each metabolite. This data generally corroborated the isotopomeric ratios that are shown in Table 2 (which had been estimated from the reduced intensity of the isotopically-normal 15-[CH$_3$] peak relative to other peaks

¶ References are given to the most complete or reliable NMR data that is currently available for these compounds; this data was used in confirming the identities of these metabolites.

in the ^{13}C NMR spectrum—due to both labelled and unlabelled forms of each metabolite in Section 2.3). Hence, this experiment provided independent confirmation of the conclusion that the extent of isotopic-labelling in both artemisinin (**1**) and arteannuin H (**21**) was significantly less than that for all the other fourteen metabolites of **2**.

3. Conclusion

Based on several precedents from the chemical literature, and from our recent detailed experimental investigations (and speculations) on the in vitro transformations of dihydroartemisinic acid,[2,4,6,42–44] it is proposed that the 16 compounds which have been isolated from feeding *A. annua* with compound **2** are biogenetically related by the series of spontaneous reactions occurring in vivo, which are shown in Scheme 3. This series of transformations is also consistent with all of the time-course studies discussed above (see in particular Figures 4 and 8).

The first chemical reaction in the in vivo transformations of dihydroartemisinic acid is the 'ene-type' addition of singlet molecular oxygen to the $\Delta^{4,5}$-double bond of **2**, which is known to yield the tertiary allylic hydroperoxide **15**, as well as to two alternative secondary allylic hydroperoxides, as minor products.[49] Most of the metabolites encountered in this study can then be derived from **15** by the established reactions of such hydroperoxides.[50] In particular, S_N2' attack of the 12-carboxylate at the allylic hydroperoxide group would result in either dihydro-*epi*-deoxyarteannuin B (**7**) or its epimer, dihydro-deoxyarteannuin B (**16**), while both dihydroartennuin B (**5**) and its lactone ring-opened analogue **17** may arise by protonation of the hydroperoxide and rearrangement to an epoxy carbocation, which accompanies the elimination of water. Spontaneous Hock cleavage[39,50] of the tertiary allylic hydroperoxide group in **15** would produce the enolic intermediate shown in both Schemes 1 and 3, which can then either tautomerize to the aldehyde metabolite **14**, or react with $^3O_2$ to yield artemisinin (**1**) and/or deoxyartemisinin (**18**).

If the metabolite **7** were to undergo an 'ene-type' reaction between its $\Delta^{4,5}$-double bond and $^1O_2$, in the same manner as that which has already been demonstrated experimentally for **2**,[39] then homolysis[50] of the resulting hydroperoxides would also account for the minor metabolites arteannuin K (**19**), arteannuin L (**20**) and arteannuin M (**11**) (there is in vitro evidence to support such a mechanism for at least one of these metabolites).[43] Known spontaneous reactions of one of the alternative secondary allylic hydroperoxides[2,4,49] from reaction of $^1O_2$ with **2** would also simply account for the biogenesis of arteannuin H (**21**) and arteannuin I (**22**), while, by analogy, the other secondary hydroperoxide might reasonably be expected to undergo a similar spontaneous conversion[2,4] to arteannuin J (**23**), although there is as yet no direct evidence for this in the literature.

As noted above, the predominant transformation pathway observed for **2** in vivo (**2**→**15**→(**5**+**7**+**14**) in Scheme 2) was qualitatively exactly the same as that which has been shown to occur for the spontaneous autoxidation of **2** in vitro.[39] Quantitatively, however, there was a significant difference in that, such transformations of dihydroartemisinic acid in organic solution were completed only after several weeks in vitro, whereas they occurred within just a few days in vivo in the aerial parts of *A. annua*. In this regard, it is interesting to note that analyses of the ^2H NMR spectra of the roots invariably showed very much more limited transformations of **2** (although the pattern was probably qualitatively quite similar). These observations are consistent with the proposal that that the primary route for transformation of **2** in vivo is by an autoxidative reaction of the $\Delta^{4,5}$-double bond with singlet oxygen ($^1O_2$), which is generally considered to be the reactive species in such processes. $^1O_2$ may be relatively abundant in the aerial parts due to the presence of plant pigments which can act as photosensitizers, but rather scarce in the roots which lack pigmentation. The suggestion that, in vivo, compound **2** undergoes rapid plant pigment-photosensitized oxidation to the tertiary allylic hydroperoxide **15**, followed by subsequent spontaneous reactions of this hydroperoxide, rather than transformations mediated by enzymes, is also consistent with the observation that the same pattern of transformations was noted in plants which were allowed to die after having being fed with the labelled precursor, as for plants which were kept alive hydroponically for several days.

All of the preceding findings have led us to the conclusion that dihydroartemisinic acid is the late biogenetic precursor to almost all of the 11,13-dihydro-amorphanes and cadinane sesquiterpenes which have been described from *A. annua* in the past, and that dihydroartemisinic acid is rapidly transformed into these metabolites in vivo by spontaneously-occurring chemical reactions rather than by enzymically-catalysed processes. The least satisfactory aspect of this unifying hypothesis, which neatly accounts for the biogenesis of many of the large number of 11,13-dihydro-amorphane and cadinane sesquiterpenes which are now known from *A. annua*, relates to artemisinin (**1**) itself. Although artemisinin has sometimes been reported to be the most abundant sesquiterpene from *A. annua* (accounting for more than 1% of the total plant weight, in some studies), the appearance of the labelled form of this compound was only ever noted at the end of the various feeding experiments which are described in Sections 2.2–2.4, and the percentage of the labelled metabolite, relative to the isotopically-normal compound (which, as discussed earlier, is believed to represent the natural product that would have been present in any case), was always much lower than that found for all the other metabolites, with the exception of arteannuin H.

In this regard, it is intriguing to note that artemisinin and arteannuin H were the only two labelled metabolites to be isolated in this study which contained intact peroxy groups (effectively the hydroperoxide precursor has been 'trapped' by a second functional group in the molecule, in both cases), whereas all the other labelled metabolites are presumed to be derived from the further reactions of such hydroperoxides (see Scheme 3). The apparent correlation between chemical structure and the extent of transformation which is undergone by the labelled precursor **2** has led us to undertake a preliminary investigation of the behaviour of the tertiary allylic hydroperoxide of dihydroartemisinic acid

(**15**) in aqueous medium rather than organic solution (although published in vitro studies[39] have conclusively shown that artemisinin (**1**) is the major product from the slow transformations of **15** in $CDCl_3$ solution, there is apparently no data concerning the reactions of **15** in water). These studies turned out to be difficult to perform on account of the poor aqueous solubility of **15** and we have found that it was only possible to characterize the products from this reaction by making a comparison with the ^1H NMR spectra of 'authentic standards' which were acquired in the same aqueous medium. However, we consistently failed to observe any conversion of **15** into **1** in water in our preliminary experiments; rather, compound **15** appears to undergo transformation within a few days into **14** in alkaline solution (in which it is more soluble) and into **7** in acidic solution; although this is somewhat of an over-simpification as these aqueous transformations of **15** were quite complex in all cases. Based on these observations, we propose that the accumulation of artemisinin (**1**) and arteannuin H (**21**) in *A. annua* might only be occurring when compound **2** undergoes autoxidation in a predominantly lipophilic environment, in which the hydroperoxide intermediates are comparatively stable. Such an environment is found in the glandular trichomes[51] on the surface of the leaves and stems. Special proteins are required to transport terpenoids[52] into the glandular trichomes, which would perhaps be the last cellular compartment to be accessed when the labelled precursor is fed via the root, thereby explaining the late appearance and low yield of the labelled forms of **1** and **21**. By contrast, the biogenesis of all the other fourteen metabolites may be occurring via homolytic cleavage and other rearrangement mechanisms, which are more characteristic of allylic hydroperoxides in an aqueous environment, i.e. predominantly in the cytoplasm of those cells which constitute the bulk plant tissues. More detailed studies of the autoxidation of **2** in aqueous solution, as well as biosynthetic experiments involving isolated glandular hairs (which it should be possible to obtain by abrasion of the leaf surface) are clearly needed in order to substantiate this hypothesis.

Although we currently favour the above explanation for the apparent anomalies in the biogeneses of **1** and **21** from **2**, as compared to most of the other 11,13-dihydro-amorphane sesquiterpenes found in *A. annua*, one could, of course, put forward several other alternatives. One possibility is that although the 14 metabolites, compounds **5**, **7**, **11**, **14**–**20** and **22**–**25** are indeed all products of spontaneous autoxidation reactions of **2** in an aqueous environment, the main biosynthetic route from **2** to **1** in vivo requires the participation of an enzyme. This enzyme might perhaps be related to the dioxygenases[53] which oxidatively cleave double bonds that have been described in the literature on diapocarotenoid biosynthesis, and, for some reason, this enzyme would not have been efficiently accessed by **2** when fed via the root in this experiment (see comments on the glandular hairs above). If this were true, then the limited accumulation of **1** observed in this paper would merely be the result of a minor (alternative) autoxidative pathway, and future experiments should reveal a 'dioxygenase-like' enzyme in *A. annua* with the appropriate activity for synthesizing the 1,2,4-trioxane ring, localized in a compartment which is relatively inaccessible to **2** when fed by the root.

A second possibility is that one of the more abundant metabolites, compounds **5**, **7** or **14** which are shown as 'products' in Scheme 2 is, in fact, an intermediate in the later stages of the biogenesis of artemisinin, and that the in vitro route for the transformation of **2** into **1** via **15** is again of only minor (or even no) importance in vivo. If this were the case, then, over the course of a much more extended experiment, one might expect to see the level of whichever of these metabolites was the true intermediate in the conversion of **2** to **1** decline at a similar rate to that of the long-term accumulation of artemisinin. Given the contradictory results reported in the literature for the biosynthetic status of **5** and **7** (see Section 1), it might be worth performing future root-feeding experiments in which the labelled forms of each of one of either **5**, **7** or **14** were administered to intact plants of *A. annua* in order to resolve this question. In addition, if any of compounds **5**, **7** or **14** were truly late intermediates of **1**, then it ought to be possible to find enzymes which catalyse the conversion of one of these natural products to artemisinin.

A third possibility, also raised in Section 1, is that the 11,13-dehydro form of dihydroartemisinic acid, artemisinic acid (**3**) is of much more significance than dihydroartemisinic acid (**2**) as an advanced precursor of artemisinin (**1**). If this were the case, then the conversion of dihydroartemisinic acid (**2**) to artemisinin (**1**) observed in this paper would again represent only a minor biosynthetic pathway, and one would hope that a future feeding experiment, in which labelled artemisinic acid (**3**) was administered to intact *A. annua* plants via the roots, would result in a much more substantial recovery of labelled artemisinin (**1**). In this regard, it is interesting to note that the 11,13-dehydro analogues[‖] of several of the labelled compounds recorded in Scheme 3, including artemisinic acid (**3**),[54] arteannuin B (**4**),[55] *epi*-deoxyarteannuin B (**6**),[4,56] the keto-aldehyde **13**,[29] deoxyarteannuin B (**26**),[4] annulide (**27**)[4,57] and isoannulide (**28**)[4,57] (Fig. 1) were all also isolated from the experiments in which *A. annua* plants had been fed with **2b**, but in no case was there any evidence for detectable deuterium labelling in these natural products. It therefore, appears that there is no significant biosynthetic pathway for desaturation of **2** at the 11,13-position to yield **3** and, by implication, its metabolites **4**, **6**, **13** and **26**–**28**. However, the presence or absence of a biosynthetic pathway for the reverse reaction in *A. annua*, i.e. the reduction of artemisinic acid (**3**) at the 11,13-dehydro position to yield dihydroartemisinic acid (**2**) (and, by implication, the 16 metabolites described herein), still remains to be determined.

4. Experimental

4.1. General

All ^1H and ^{13}C NMR experiments were recorded on either a Bruker DRX 500 or an AV 600 instrument. Chemical shifts are expressed in ppm (δ) relative to TMS as internal standard. ^2H NMR spectra were recorded at 76.77 MHz in

[‖] Again, references are given to the most complete or reliable NMR data which is currently available for these natural products, which was used in establishing their identities.

$CHCl_3$ solution containing C_6D_6 (10 μl/100 ml) which acted as both an internal ^2H reference (δ_D 7.43 ppm; spectra also showed a small peak for natural abundance ^2H in the $CHCl_3$ solvent at δ_D 7.28 ppm) and as an internal standard for calibrating the amount of ^2H label present in the crude plant extracts (the deuterium integral for the d_6-benzene resonance at δ_D 7.43 ppm had the same value as that for the 15-position (δ_D 1.63 ppm) of **2a** when 0.53 mg was dissolved in 0.6 ml $CHCl_3/C_6D_6$ solution; for **2b**, 0.38 mg in 0.6 ml $CHCl_3/C_6D_6$ solution gave the same result). The chemical shift of the reference compound was considered unlikely to interfere with the products of metabolism of dihydroartemisinic acid, for which the ^2H signal was observed either in the aliphatic region (δ_D 1–2.5 ppm) or in the alkene region (δ_D 4.5–5.5 ppm) of the spectrum—see Sections 2.2 and 2.3). The ratio of the ^2H integral for all the metabolites present in the crude plant extract to the ^2H integral for the d_6-benzene internal standard was routinely used to estimate the amount of ^2H label which was present in such extracts, based on prior experiments using known amounts of the labelled precursors **2a** or **2b** (see above). In order to acquire ^2H NMR spectra, the broadband nucleus was set to the ^2H frequency and the lock cable was physically disconnected from the probe of the NMR spectrometer, so as to prevent the leakage of the lock signal into the acquisition channel. Since it then became impossible to shim the sample, our normal practice was to have first shimmed an NMR tube of the same manufacture and containing exactly the same volume of $CDCl_3$ solution (0.6 ml) immediately prior to the acquisition of each ^2H NMR spectrum. Using this technique, the linewidth at half height for most ^2H spectra was found to be fairly constant (between 2.5–3.5 Hz); we believe that the major contribution to line broadening in ^2H NMR spectra was then from the unavoidable effects of quadrupolar relaxation in ^2H NMR spectroscopy,[58,59] rather than from magnetic inhomogeneities associated with poor shimming. $^{13}C-^2H$ COSY NMR spectra were acquired in this same solvent and according to the procedures described in Refs. 44 and 45. High-resolution MS were recorded in EI mode at 70 eV on a Finnigan-MAT 95 MS spectrometer. Column chromatography (CC) was performed using silica gel 60–200 μm (Merck). HPLC separations were performed using a Varian chromatograph equipped with RI star 9040 and UV 9050 detectors and a normal phase semi-preparative YMC diol column (20 mm×25 cm), flow rate 8 ml/min.

4.1.1. *A. annua* plants. Several batches of *A. annua* plants were grown in individual pots outdoors in Hong Kong, from seed which had been sown between October and February (seeds were supplied by the NCCPG *Artemisia* collection, Cambridge, UK, and taxonomically verified specimens are held by the collection). These plants were generally used in feeding experiments when mature and beginning to come into flower (3–4 months after germination).

4.1.2. Determination of the optimum method for feeding [15-$^{13}C^2H_3$]-dihydroartemisinic acid (2a) to *A. annua* plants

4.1.2.1. Preparation of the feeding solution of 2a. The maximum solubility of compound **2a** in TRIS buffer (50 mM, pH 8.2) was determined as ca. 2.3 mg/ml after ultrasonification for 1 h (^1H NMR analysis showed no changes in the chemical composition of **2a** as a result of ultrasonification). In consequence, feeding solutions were prepared containing 2.0–2.2 mg **2a**/1 ml of buffer.

4.1.2.2. Procedure for 'stem-feeding' cut plants. Individual plants (3–5 g) were cut just above the level of the soil and were fed with the labelled precursor at a rate of approximately 1 mg **2a**/2 g fresh plant material, by immersing the cut stem in a vial containing the feeding solution. The plants were exposed to a strong light source (500 W bulb) and a constant flow of air (from a nearby fan) in order to promote the uptake of the feeding solution via the transpiration stream. Under these conditions, plants were normally able to take up all the feeding solution over a period of 2–6 h. A small amount of TRIS buffer was then added to the feeding vial in order to rinse any remaining labelled precursor from the walls of the vial, and the plants were left for a short while longer to take up this additional small volume.

4.1.2.3. Procedure for feeding intact plants via the root. Individual plants (3–5 g) were carefully removed from the excess soil in their pots and their roots were washed free of remaining soil. The cleaned root was immersed in a vial containing the feeding solution (approximately 1 mg of **2a**/2 mg fresh weight plant material) and exposed to strong light and air flow, as above, in order to increase the rate of transpiration. All the feeding solution was normally assimilated by intact plants within 4–6 h under these conditions.

4.1.2.4. Extraction procedure. For both cut (stem-fed) and intact (root-fed) plants, it was found that the total plant weight after complete assimilation of the feeding solution was between 60–80% of the original weight just before feeding; this loss in weight was probably due to some dehydration occurring as a result of the forcing conditions which were employed to stimulate transpiration. Cut plants were separated into stems and leaf/flower compartments and these two compartments were extracted separately; intact plants were separated into stem, leaf/flower and root compartments. Each portion was ground to a fine powder under liquid N_2, then immersed in CH_2Cl_2 (ca. 250 ml/10 g plant material) in a container wrapped in aluminium foil (to exclude light and thereby minimize the formation of autoxidation products from **2**) and left overnight on a shaker. The solvent was dried ($MgSO_4$) and removed by rotary evaporation to yield the crude plant extract as an aromatic green gum; this procedure was repeated three times and the dichloromethane extracts (stems 1–3% w/w; leaf/flower 2–5% w/w; root <0.5% w/w) were combined and analysed immediately by NMR. A second methanolic extract of the residual plant material was then made in the same way, using MeOH in place of CH_2Cl_2.

4.1.2.5. Analysis of the CH_2Cl_2 extracts. The initial analysis of the CH_2Cl_2 extracts of *A. annua* plants which had been fed with **2a** was made by ^2H NMR spectroscopy and samples were dissolved in $CHCl_3$ (0.6 ml) containing C_6D_6 (10 μl/100 ml), as in Section 4.1. Samples were also sometimes analysed by ^1H and ^{13}C NMR spectroscopies in $CDCl_3$ solution in order to confirm the results of ^2H NMR analysis. However, these analyses were in general much less informative, as signals from the plant matrix obscured most of the resonances from the labelled precursor (by contrast, there was negligible signal from the plant matrix in the ^2H NMR spectra of the crude extracts and the analyses of these

spectra for the presence of the labelled precursor and/or its metabolites was a straightforward matter).

4.1.2.6. Analysis of the MeOH extracts. The MeOH extracts from plants fed with **2a** were dissolved in MeOH and analysed by ^{2}H NMR sectroscopy. Generally, such analyses consistently indicated a much lower incorporation of ^{2}H-label (<15% of that present in the CH_2Cl_2 extracts) and the methanolic extracts were not investigated further in this experiment or any of the experiments described in subsequent sections.

4.1.3. Time-course study of the transformation of 2a which was fed to intact *A. annua* plants via the root. Twenty one plants (typical weight 5–10 g per plant) were fed individually via the root with 2.5 ml of a stock solution of **2a** in TRIS buffer (105 mg/52.5 ml), as described in Section 4.1.2. Uptake of the labelled precursor was completed within 6 h of the commencement of feeding and plants were kept alive under hydroponic conditions for several days by continual replenishment of deionized water into the feeding vials. The ambient temperature was 22–28 °C over the course of the experiment with mostly overcast weather. Groups of three plants were separated into aerial (stem/leaf/flower) and root compartments and extracted individually by CH_2Cl_2 (see previous section) at 10, 24, 33, 48, 72, 96 and 144 h after the commencement of feeding. Immediate analysis was made, as previously, by ^{2}H NMR spectroscopy following preparation of each of the 21 crude aerial CH_2Cl_2 extracts and 21 root extracts. The incorporation of ^{2}H label into the CH_2Cl_2 extract of the aerial parts remained at 1.5–3 mg (30–60%) over the course of the experiment, while the incorporation into the roots dropped from 15% at the beginning to almost 0% at the end. The percentage amount of each metabolite in each extract which was present in the aliphatic region of the spectrum was then computationally modeled by a programme written in Microsoft Excel. This simulation calculated the intensity of 151 discrete points, at intervals of 0.01 ppm over the range δ_D 1.0–2.5 ppm, as a summation of each component doublet in the spectrum, for which a discrete Lorentzian line was calculated according to the formula:[60]

$$\mathrm{Int}_i = A\ (1/T_2)/[(1/T_2)^2 + 4\pi^2(\nu_i - \nu_a)^2]$$

where: Int_i=intensity of discrete frequency point i; A=weighting factor (equivalent to the percentage amount of a metabolite in the crude extract); $1/T_2$=line broadening factor (in fact, the reciprocal of the transverse relaxation rate—for clearly resolved peaks such as δ_D 7.43 [d_6-benzene] it could be obtained by measuring the line width at half height in the real spectrum and multiplying by π); ν_i=chemical shift in Hz of discrete point at frequency i, which is being simulated; ν_a=two frequencies calculated per metabolite, corresponding to both lines of a doublet, centred at the appropriate resonant frequency for the chemical shift at the 15-position of that metabolite, expressed in Hz (B_0=76.77 MHz) and separated by 19.1 Hz.

This spectral simulation programme allowed the operator to enter parameters for the line width; the precise value for the center of each of the doublet resonances; and the estimated percentage composition of the major doublet resonances, for metabolites which were centred at δ_D 1.27 (**15a**), 1.34 (**5a** and possibly **17a**), 1.63 (**2a**), 1.68 (**7a** and possibly **16a**) and 2.14 (**14a**) ppm (as well as minor doublet resonances at 1.31 (**24a**), 1.32 (**25a**), 1.39 (**11a**), 1.44 (**1a**), 1.51 (**18a**), 1.78 (**19a** and **23a**) and 2.19 (unidentified) ppm. These parameters could then be repetitively re-adjusted in order to obtain optimum agreement between the simulated spectrum and the ^{2}H NMR experiment.

4.1.3.1. Analysis of extracts from feeding of 2a by ^{13}C–^{2}H COSY. See Refs. 44 and 45 for a description of the ^{13}C–^{2}H COSY experiment. Several samples which appeared to be similar by one-dimensional ^{2}H NMR were generally combined together, giving extracts totalling 100–150 mg in weight—the maximum amount which could be dissolved in 0.2 ml of $CHCl_3$ in a 'capillary' NMR tube and then analysed using a 'dual' microprobe.

4.1.4. Time-course study of the transformation of 2b which was fed to intact *A. annua* plants via the root. Forty five plants (5–10 g fresh weight for each plant) were fed individually via the root with 2.2 ml of a stock solution of **2b** in TRIS buffer (200 mg/100 ml) as described in the preceding sections. Uptake of the labelled precursor was completed within 4–10 h of the commencement of feeding. Three plants were then extracted immediately to serve as a 'zero-time' point and the remaining plants were divided into two groups of 21 plants each.[**] One group was kept alive hydroponically for several days by periodically replenishing deionized water in the feeding vials, while the other was allowed to die by dessication. Ambient temperature over the course of the experiment was 30–32 °C, with strong sunlight throughout.

Three plants from each group were then separated into aerial (stem/leaf/flower) and root compartments and extracted individually by CH_2Cl_2 (as previously) on an evening/morning schedule at 8/24, 32/48, 56/72 and 80 h after the 'zero-time' point. The residual plant material was subsequently re-extracted by MeOH: as previously, the MeOH extracts contained comparatively little ^{2}H-label relative to the CH_2Cl_2 extracts and were not analysed further. Immediate ^{2}H NMR analysis of each of the 45 CH_2Cl_2 aerial extracts revealed that the recovery of ^{2}H label remained constant at between 1–2 mg from the aerial parts (20–40%) over the course of the experiment. The percentage amount for each of the most dominant labelled components of the mixture (resonances at δ_D 1.29 (**15b**), 1.34 (**5b** and **17b**), 1.63 (**2b**), 1.68 (**17b** and **16b**) and 2.14 (**14b**) ppm) was then computationally simulated by a programme written in Microsoft Excel which was similar to that described in Section 4.1.3, but in which each metabolite resonance was represented by a singlet rather than a doublet. Minor metabolites, represented by singlets at 1.31 (**24b**), 1.32 (**25b**), 1.39 (**11b**), 1.44 (**1b**), 1.52 (**18b**), 1.78 (**19b** and **23b**) and 2.19 (unidentified) ppm, were also included in these simulations in order to obtain better fits to the experimental data. Strongly overlapped and poorly resolved ^{2}H-resonances were also observed between δ_D 4.7

[**] In a control experiment, vials containing **2b** in TRIS buffer (but without *A. annua* plants) were subjected to these same conditions over the duration of the feeding period. No changes were noted in the composition of **2b**.

and 5.1 ppm, but generally accounted for less than 5% of the total ^{2}H-incoroporation by a comparison of the ^{2}H integral for this region with that of the aliphatic region. No attempt was made to include these resonances, which are probably due to metabolites **20b**, **21b** and **22b**, in the quantification.

4.2. Separation of labelled metabolites from the CH_2Cl_2 extracts of the aerial parts of *A. annua* plants which were fed with 2b

All 45 CH_2Cl_2 extracts from the aerial parts of *A. annua* plants which had been fed with **2b** were combined together (total weight 1.7 g) and subjected to a preliminary HPLC separation (18% EtOAc/*n*-hexane) from which 26 fractions were collected over a 45 min period. The column was 'washed' with 50% EtOAc/*n*-hexane/1% AcOH between each injection in order to obtain a 'polar residue'. Individual fractions from the preliminary HPLC separation, which showed significant signal in ^{2}H NMR spectroscopy, were then subjected to further HPLC separations, with the composition of the mobile phase being varied according to the degree of polarity of each fraction under study, in order to obtain labelled metabolites of sufficient purity for spectroscopic characterization. The compounds 'washed' from the preliminary HPLC and collected as the 'polar residue' were also re-subjected to HPLC separation (50% EtOAc/*n*-hexane/1% AcOH) and 24 fractions were collected. Those fractions which contained resonances in their ^{2}H NMR spectra, corresponding to a more polar series of metabolites, were subjected to yet more rounds of HPLC separation until pure compounds were obtained. The 16 labelled metabolites which were isolated from all these extensive HPLC procedures are listed below.

4.2.1. [15-C^{2}H$_3$]-Artemisinin (1b)/artemisinin (1). Oil (9.0 mg; R_t 19.2 min in the preliminary HPLC; R_t 29.2 min, 10% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.86 (1H, s, H-5), 3.40 (1H, dq, J=5.5, 7.3 Hz, H-11), 2.44 (1H, ddd, J=14.6, 13.0, 3.9 Hz, H-3), 2.05 (1H, ddd, J=14.6, 4.6, 2.7 Hz, H-2), 2.00 (1H, ddddd, J=13.9, 2.9, 2.9, 2.9, 2.7 Hz, H-2), 1.88 (1H, dddd, J=10.5, 3.4, 3.4, 3.4 Hz, H-8), 1.77 (2H, m, H-7, H-9) 1.49 (1H, m, H-10) 1.45 (>95% of [3H], s, H-15), 1.38 (1H, m, H-1), 1.21 (3H, d, J=7.3 Hz, H-13), 1.08 (1H, dddd, J=12.8, 12.8, 12.8, 2.5 Hz, H-8), 1.00 (3H, d, J=5.9 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 172.1 (C-12), 105.4 (C-4), 93.7 (C-5), 79.5 (C-6), 50.1 (C-1), 45.0 (C-7), 37.5 (C-10), 35.9 (C-3), 33.6 (C-9), 32.9 (C-11), 25.2 (C-15, >95% intensity of isotopically-normal peak), 24.9 (C-2), 23.4 (C-8), 19.8 (C-14), 12.6 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.44 (s); CIMS *m*/*z* (rel. int.): 253 [M^+-O_2, $C_{15}H_{19}{}^2H_3O_3$] (2)/250 [M^+-O_2, $C_{15}H_{22}O_3$] (26), 235 (15), 232 (17), 209 (32), 192 (100), 180 (34), 152 (72).

4.2.2. [15-C^{2}H$_3$]-Dihydroartemisinic acid (2a)/dihydroartemisinic acid (2). Oil (1.0 mg; R_t 25.2 min in the preliminary HPLC; R_t 15.6 min, 12% EtOAc/*n*-hexane/0.5% AcOH). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.11 (1H, s, H-5), 2.51 (2H, m, H-6, H-11), 1.98–1.87 (2H, m, H-2, H-3), 1.80 (1H, dd, J=17.1, 6.9 Hz, H-3), 1.63 (<60% of [3H], s, H-15), 1.19 (3H, d, J=6.9 Hz, H-13), 1.11 (1H, dddd, J=12.8, 12.8, 12.3, 3.2 Hz, H-8), 0.96 (1H, dddd, J=12.3, 12.3, 12.3, 2.7 Hz, H-9), 0.87 (3H, d, J=6.4 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 182.0 (C-12), 135.9 (C-4), 119.3 (C-5) 43.6 (C-7), 42.3 (C-11), 41.7 (C-1), 36.3 (C-7), 35.3 (C-9), 27.7 (C-10), 27.4 (C-8), 26.6 (C-3), 25.8 (C-2), 23.8 (C-15, <10% intensity of the isotopically-normal peak), 19.7 (C-14), 15.1 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.63 (s); HREIMS *m*/*z* (rel. int.): 239.1966 [M^+, $C_{15}H_{21}{}^2H_3O_2$ requires 239.1964] (10)/236.1767 [M^+, $C_{15}H_{24}O_2$ requires 236.1776] (2), 211 (2), 166 (27), 165 (100), 162 (16).

4.2.3. [15-C^{2}H$_3$]-Dihydroarteannuin B (5b)/dihydroarteannuin B (5). Oil (0.6 mg; R_t 25.2 min in the preliminary HPLC; R_t 33.0 min, 12% EtOAc/*n*-hexane/0.5% AcOH). ^{1}H NMR (δ, $CDCl_3$) ppm: 3.02 (1H, s, H-5), 2.77 (1H, dq, J=8.5, 8.0 Hz, H-11), 2.25 (1H, ddd, J=13.0, 8.5, 2.5 Hz, H-7), 1.93 (1H, dd, J=14.4, 4.8 Hz, H-3), 1.88 (1H, m, H-9), 1.82 (1H, dddd, J=12.8, 2.8, 2.8, 2.8 Hz, H-8), 1.54–1.45 (3H, m, H-2, H-8, H-10), 1.38 (3H, d, J=8.0 Hz, H-13), 1.35 (ca. 30% of [3H], s, H-15), 1.23 (1H, dddd, J=12.3, 12.3, 12.3, 4.1 Hz, H-9), 0.96 (3H, d, J=6.6 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 179.5 (C-12), 83.0 (C-6), 59.5 (C-5), 58.1 (C-4), 50.2 (C-7), 45.6 (C-1), 38.6 (C-11), 34.8 (C-9), 30.5 (C-10), 24.1 (C-3), 23.1 (C-15, ca. 30% intensity of the isotopically-normal peak), 21.6 (C-8), 18.5 (C-14), 16.2 (C-2), 12.6 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.35 (s).

4.2.4. [15-C^{2}H$_3$]-Dihydro-*epi*-deoxyarteannuin B (7b)/dihydro-*epi*-deoxyarteannuin B (7). Oil. (3.8 mg; R_t 12.5 min in the preliminary HPLC; R_t 17.6 min, 7% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.64 (1H, s, H-5), 3.14 (1H, dq, J=7.3, 7.1 Hz, H-11), 2.15–2.06 (2H, m, H-3, H-7), 2.04 (1H, dddd, J=18.1, 11.2, 6.6, 2.3 Hz, H-3), 1.89 (1H, dddd, J=13.0, 6.6, 2.7, 1.4 Hz, H-2), 1.75–1.63 (3H, m, H-2, H-8, H-9), 1.69 (<30% of [3H], s, H-15), 1.43 (1H, dddq, J=10.5, 10.5, 6.9, 6.4 Hz, H-10), 1.19 (1H, m, H-8), 1.15 (3H, d, J=7.1 Hz, H-13), 1.05 (1H, dddd, J=12.6, 10.5, 9.8, 2.5 Hz, H-9), 0.94 (3H, d, J=6.4 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 179.4 (C-12), 142.2 (C-4), 121.8 (C-5), 83.2 (C-6), 46.6 (C-1), 42.8 (C-7), 39.7 (C-11), 32.5 (C-9), 30.8 (C-3), 29.7 (C-10), 23.5 (C-15; ca. 40% intensity of the the isotopically-normal peak), 23.4 (C-8), 21.0 (C-2), 19.6 (C-14), 9.5 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.68 (s); HREIMS *m*/*z* (rel. int.): 237.1812 [M^+, $C_{15}H_{19}{}^2H_3O_2$ requires 237.1808] (38)/234.1623 [M^+, $C_{15}H_{22}O_2$ requires 234.1620] (13), 219 (12), 193 (75), 190 (23), 164 (100), 161 (37).

4.2.5. [15-C^{2}H$_3$]-Arteannuin M (11b)/arteannuin M (11). Oil (0.1 mg; R_t 17.0 min for the 'polar residue' from preliminary HPLC; R_t 40.0 min, 40% EtOAc/*n*-hexane/0.7% AcOH). ^{1}H NMR (δ, $CDCl_3$) ppm: 3.46 (1H, s, H-5), 3.09 (1H, dq, J=7.1, 7.2 Hz, H-11), 2.65 (1H, ddd, J=12.8, 7.1, 5.5 Hz, H-7), 1.39 (<50% of [3H], s, H-15), 1.13 (3H, d, J=7.2 Hz, H-13), 0.93 (3H, d, J=6.4 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 179.1 (C-12, not seen due to low intensity), 86.3 (C-6, not seen due to low intensity), 74.2 (C-5), 72.6 (C-4, not seen due to low intensity), 41.7 (C-1), 39.1 (C-7), 38.8 (C-11), 34.2 (C-3), 32.3 (C-9), 29.9 (C-10), 26.6 (C-15, ca. 50% intensity of the other peaks), 23.9 (C-8), 22.1 (C-2), 20.1 (C-14), 9.4 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.39 (s); HREIMS *m*/*z* (rel. int.): 253.1751 [M^+-H_2O, $C_{15}H_{19}{}^2H_3O_3$ requires 253.1757] (3)/250.1553 [M^+-H_2O, $C_{15}H_{22}O_3$ requires 250.1569] (1), 235 (4), 232 (1), 225 (10), 222 (6), 195 (12), 179 (100).

4.2.6. [15-C^2H_3]-1α-Formyl-2β-[3-butanone]-3α-methyl-6β-(2-propanoic acid)-cyclohexane (14b)/1α-formyl-2β-[3-butanone]-3α-methyl-6β-(2-propanoic acid)-cyclohexane (14). Oil (0.3 mg). ^1H NMR (δ, $CDCl_3$) ppm: 9.58 (1H, d, *J*=4.9 Hz, H-5), 2.45–2.32 (3H, m, H-3, H-6), 2.14 (ca. 30% of [3H], s, H-15), 1.18 (3H, d, *J*=6.9 Hz, H-13), 0.94 (3H, d, *J*=6.4 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 208.3 (C-4), 205.9 (C-5), 177.4 (C-12), 56.1 (C-6), 42.3 (C-1), 41.2 (C-11), 41.0 (C-7), 38.1 (C-3), 34.7 (C-9), 33.4 (C-10), 30.0 (C-15, ca. 30% intensity of the isotopically-normal peak), 26.7 (C-2), 23.5 (C-8) 19.6 (C-14), 14.4 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 2.13 (s); HREIMS *m/z* (rel. int.): 253.1749 [M^+-H_2O, $C_{15}H_{19}{}^2H_3O_3$ requires 253.1757] (3)/250.1564 [M^+-H_2O, $C_{15}H_{22}O_3$ requires 250.1569] (1), 235 (76), 232 (31), 222 (6), 179 (100).

4.2.7. [15-C^2H_3]-Dihydroartemisinic acid tertiary hydroperoxide (15b)/dihydroartemisinic acid tertiary hydroperoxide (15). Oil (0.8 mg; R_t 9.8 for the 'polar residue' from preliminary HPLC; R_t 26.6 min, 22% EtOAc/*n*-hexane/0.5% AcOH). ^1H NMR (δ, $CDCl_3$) ppm: 5.25 (1H, s, H-5), 2.75 (1H, dq, *J*=6.9, 6.9 Hz, H-11), 1.29 (<30% of [3H], s, H-15), 1.27 (3H, d, *J*=6.9 Hz, H-13), 0.94 (3H, d, *J*=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 178.4 (C-12), 146.4 (C-6), 120.2 (C-5), 80.8 (C-4), 47.2 (C-7), 45.0 (C-1), 40.6 (C-11), 38.6 (C-10), 35.5 (C-9), 32.7 (C-8), 28.8 (C-3), 24.5 (C-15, ca. 25% intensity of the isotopically-normal peak), 22.5 (C-2), 20.0 (C-14), 15.8 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 1.28 (s).

4.2.8. [15-C^2H_3]-Dihydro-deoxyarteannuin B (16b)/dihydro-deoxyarteannuin B (16). Oil (0.1 mg; R_t 13.8 min in the preliminary HPLC; R_t 26.6 min, 5% EtOAc/*n*-hexane). ^1H NMR (δ, $CDCl_3$) ppm: 5.53 (1H, s, H-5), 2.74 (1H, dq, *J*=8.2, 8.0 Hz, H-11), 2.19 (1H, ddd, *J*=12.3, 8.2, 2.3 Hz, H-7), 2.03 (1H, m, H-3), 1.92 (1H, m, H-3) 1.80 (1H, m, H-9) 1.70 (<30% of [3H], s, H-15), 1.67 (1H, m, H-8), 1.44 (1H, dddd, *J*=12.3, 12.3, 12.3, 3.4 Hz, H-8), 1.34 (3H, d, *J*=8.0 Hz, H-13), 1.17 (1H, dddd, *J*=12.3, 12.1, 12.1, 3.7 Hz, H-9), 0.97 (3H, d, *J*=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 180.6 (C-12, not seen due to low intensity), 142.3 (C-4), 120.5 (C-5), 86.7 (not seen due to low intensity), 49.8 (C-7), 46.6 (C-1), 39.0 (C-11), 35.3 (C-9), 30.7 (C-10), 26.0 (C-3), 24.1 (C-15, <20% intensity of the other peaks), 22.0 (C-8), 20.1 (C-2), 19.9 (C-14), 13.1 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 1.68 (s); HREIMS *m/z* (rel. int.): 237.1811 [M^+, $C_{15}H_{19}{}^2H_3O_2$ requires 237.1808] (47)/234.1623 [M^+, $C_{15}H_{22}O_2$ requires 234.1620] (10), 193 (50), 191 (52), 164 (100), 161 (28).

4.2.9. [15-C^2H_3]-4α,5α-Epoxy-6α-hydroxy-amorphan-12-oic acid (17b)/4α,5α-epoxy-6α-hydroxy-amorphan-12-oic acid (17). Oil (0.3 mg; R_t 10.6 min for the 'polar residue' from preliminary HPLC; R_t 22.1 min, 22% EtOAc/*n*-hexane/0.6% AcOH). ^1H NMR (δ, $CDCl_3$) ppm: 3.24 (1H, s, H-5), 3.06 (1H, dq, *J*=4.0, 7.1 Hz, H-11), 1.36 (<30% of [3H], s, H-15), 1.33 (3H, d, *J*=7.1 Hz, H-13), 0.87 (3H, d, *J*=6.8 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 181.1 (C-12), 70.6 (C-6), 60.9 (C-4, C-5), 50.1 (C-7), 48.6 (C-1), 39.5 (C-11), 34.6 (C-9), 30.7 (C-10), 26.2 (C-8), 24.4 (C-3), 23.5 (C-15, ca. 30% intensity of the isotopically-normal peak), 19.0 (C-14), 18.9 (C-13), 15.1 (C-2); ^2H NMR (δ, $CHCl_3$) ppm: 1.35 (s); HREIMS *m/z* (rel. int.): 253.1750 [M^+-H_2O, $C_{15}H_{19}{}^2H_3O_3$ requires 253.1757] (4)/250.1565 [M^+-H_2O, $C_{15}H_{22}O_3$ requires 250.1569] (1), 235 (4), 232 (1), 179 (100).

4.2.10. [15-C^2H_3]-Deoxyartemisinin (18b)/deoxyartemisinin (18). Oil (1.5 mg; R_t 15.8 min in the preliminary HPLC; R_t 23.4 min, 10% EtOAc/*n*-hexane). ^1H NMR (δ, $CDCl_3$) ppm: 5.70 (1H, s, H-5), 3.19 (1H, dq, *J*=4.6, 7.1 Hz, H-11), 2.01 (1H, ddd, *J*=13.1, 4.6, 4.6 Hz, H-7), 1.94–1.88 (2H, m, H-2, H-8), 1.63 (1H, m, H-3), 1.53 (<50% of [3H], s, H-15), 1.20 (3H, d, *J*=7.1 Hz, H-13), 0.94 (3H, d, *J*=5.7 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 173.5 (C-12), 109.2 (C-4), 99.7 (C-5), 82.4 (C-6), 44.6 (C-1), 42.4 (C-7), 35.4 (C-10), 33.9 (C-3), 33.5 (C-9), 32.8 (C-11), 24.0 (C-15, ca. 30% intensity of the isotopically-normal peak), 23.5 (C-8), 22.0 (C-2), 18.6 (C-14), 12.6 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 1.52 (s); HREIMS *m/z* (rel. int.): 269.1699 [M^+, $C_{15}H_{19}{}^2H_3O_4$ requires 269.1707] (10)/266.1509 [M^+, $C_{15}H_{22}O_4$ requires 266.1518] (6), 225 (35), 222 (10), 168 (63), 165 (52), 164 (100), 151 (77).

4.2.11. [15-C^2H_3]-Arteannuin K (19b)/arteannuin K (19). Oil (0.1 mg; R_t 35–39 min in the preliminary HPLC; R_t 35.1 min, 20% EtOAc/*n*-hexane). ^1H NMR (δ, $CDCl_3$) ppm: 5.67 (1H, d, *J*=3.2 Hz, H-3), 3.69 (1H, d, *J*=3.2 Hz, H-5), 3.11 (1H, dq, *J*=6.8, 7.1 Hz, H-11), 2.72 (1H, ddd, *J*=10.7, 6.8, 5.8 Hz, H-7), 2.29 (1H, ddd, *J*=18.5, 11.5, 3.2 Hz, H-2), 1.98 (1H, m, H-2), 1.78 (<40% of [3H], s, H-15), 1.15 (3H, d, *J*=7.1 Hz, H-13), 0.94 (3H, d, *J*=5.8 Hz, H-14); ^2H NMR (δ, $CHCl_3$) ppm: 1.78 (s); HREIMS *m/z* (rel. int.): 253.1756 [M^+, $C_{15}H_{19}{}^2H_3O_3$ requires 253.1757] (3)/250.1557 [M^+, $C_{15}H_{22}O_3$ requires 250.1569] (1), 208 (30), 180 (27), 167 (100).

4.2.12. [15-C^2H_2]-Arteannuin L (20b)/arteannuin L (20). Oil (0.1 mg; R_t 35–39 min in the preliminary HPLC; R_t 32.8 min, 20% EtOAc/*n*-hexane). ^1H NMR (δ, $CDCl_3$) ppm: 4.94 (33% of [1H], dd, *J*=1.6, 1.6 Hz, H-15), 4.91 (33% of [1H], dd, *J*=1.6, 1.6 Hz, H-15), 4.11 (1H, s, H-5), 3.11 (1H, dq, *J*=5.9, 7.1 Hz, H-11), 2.60 (1H, ddd, *J*=11.4, 5.9, 5.9 Hz, H-7), 2.38 (1H, ddd, *J*=14.6, 13.5, 5.1 Hz, H-3), 2.26 (1H, dd, *J*=14.6, 4.1 Hz, H-3), 1.86 (1H, d, *J*=12.6 Hz, H-2), 1.75 (1H, m, H-8), 1.72 (1H, ddd, *J*=11.2, 11.2, 3.5 Hz, H-1), 1.63 (1H, m, H-9), 1.45 (1H, dddd, *J*=13.5, 12.6, 11.2, 4.1 Hz, H-2), 1.14 (3H, d, *J*=7.1 Hz, H-13), 1.09 (1H, ddd, *J*=12.8, 12.8, 12.8 Hz, H-9) 0.95 (3H, d, *J*=6.6 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 179.2 (C-12, not seen due to low intensity), 146.6 (C-4, not seen due to low intensity), 114.9 (C-15, <20% intensity of the isotopically-normal peak); 86.2 (C-6, not seen due to low intensity), 73.5 (C-5), 41.8 (C-1), 38.8 (C-11), 38.4 (C-7), 32.4 (C-9), 30.7 (C-10), 29.1 (C-3), 25.2 (C-2), 24.3 (C-8), 20.1 (C-14), 9.3 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 4.94 (br s); HREIMS *m/z* (rel. int.): 252.1687 [M^+, $C_{15}H_{20}{}^2H_2O_3$ requires 252.1695] (4)/250.1564 [M^+, $C_{15}H_{22}O_3$ requires 250.1569] (1), 234 (8), 232 (3), 179 (100), 178 (48), 177 (32).

4.2.13. [15-C^2H_2]-Arteannuin H (21b)/arteannuin H (21). Oil (0.3 mg; R_t 9.2–11.2 min in the preliminary HPLC; R_t 14.3 min, 2% EtOAc/*n*-hexane). ^1H NMR (δ, $CDCl_3$) ppm: 5.03 (1H, d, *J*=12.3 Hz, H-5), 4.91 (84% of

[1H], q, J=1.8 Hz, H-15), 4.84 (85% of [1H], q, J=1.8 Hz, H-15), 3.52 (1H, q, J=7.1 Hz, H-11), 2.24 (1H, ddd, J=13.9, 4.5, 2.9 Hz, H-3), 2.18 (1H, dd, J=13.9, 13.9 Hz, H-3), 2.08 (1H, ddd, J=12.3, 4.2, 3.9 Hz, H-6), 1.95 (1H, dddd, J=13.7, 4.1, 2.1, 2.1 Hz, H-2), 1.87 (1H, dddd, J=13.2, 3.5, 3.5, 3.5 Hz, H-9), 1.77 (1H, ddd, J=14.0, 3.7, 3.1 Hz, H-8), 1.74 (1H, dddd, J=12.0, 3.9, 3.7, 1.0 Hz, H-7), 1.64 (1H, m, H-10), 1.38 (1H, dddd, J=13.9, 13.7, 4.7, 4.5 Hz, H-2), 1.26 (1H, m, H-8), 1.21 (3H, d, J=7.1 Hz, H-13), 1.05 (1H, dddd, J=13.2, 12.3, 12.3, 3.0 Hz, H-9), 0.88 (3H, d, J=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 180.1 (C-12), 145.3 (C-4), 106.6 (C-15; no clear change in peak intensity relative to the isotopically-normal spectrum), 84.8 (C-5), 50.9 (C-6), 45.2 (C-1), 43.3 (C-7), 40.3 (C-11), 35.5 (C-9), 30.7 (C-3), 28.6 (C-2 and C-10), 22.7 (C-8), 19.7 (C-14), 15.6 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 4.93 (br s), 4.85 (br s) HREIMS m/z (rel. int.): 252.1690 [M^+, $C_{15}H_{20}{}^2H_2O_3$ requires 252.1695] (16)/250.1575 [M^+, $C_{15}H_{22}O_3$ requires 250.1569] (80), 234 (48), 232 (32), 206 (77), 204 (33), 191 (25), 179 (60), 177 (100).

4.2.14. [15-C^{2}H$_2$]-Arteannuin I (22b)/arteannuin I (22). Oil (1.1 mg; R_t 13.1 min in the preliminary HPLC; R_t 21.3 min, 6% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.05 (27% of [1H], s, H-15), 4.96 (1H, d, J=11.9 Hz, H-5), 4.82 (27% of [1H], s, H-15), 2.69 (1H, dq, J=7.1, 7.3 Hz, H-11), 2.25 (1H, d, J=13.9 Hz, H-3), 2.15 (1H, ddd, J=13.9, 13.9, 4.6 Hz, H-3), 2.04–1.95 (2H, m, H-2, H-6) 1.94–1.86 (2H, m, H-7, H-9) 1.81–1.73 (2H, m, H-1, H-8), 1.47 (1H, dddd, J=13.9, 13.9, 4.5, 4.5 Hz, H-2), 1.29 (1H, dddd, J=13.1, 13.1, 13.1, 3.7 Hz, H-8), 1.23 (3H, d, J=7.3 Hz, H-13), 1.05 (1H, dddd, J=13.2, 13.2, 13.1, 3.9 Hz, H-9) 0.91 (3H, d, J=6.4 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 174.4 (C-12), 146.0 (C-4), 105.1 (C-15; ca. 20% intensity of the isotopically-normal peak); 76.8 (C-5), 45.5 (C-6), 43.7 (C-1), 40.5 (C-11), 40.0 (C-7), 35.0 (C-9), 29.4 (C-3), 28.7 (C-2), 28.1 (C-10), 22.7 (C-8), 20.1 (C-14), 13.3 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 5.07 (s), 4.86 (s); HREIMS m/z (rel. int.): 236.1739 [M^+, $C_{15}H_{20}{}^2H_2O_2$ requires 236.1746] (100)/234.1614 [M^+, $C_{15}H_{22}O_2$ requires 234.1619] (33), 208 (73), 206 (22), 191 (24), 124 (100).

4.2.15. [15-C^{2}H$_3$]-Arteannuin J (23b)/arteannuin J (23). Oil (0.1 mg—only ca. 50% pure; R_t 13.1 min in the preliminary HPLC; R_t 20.4 min, 6% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.37 (1H, s, H-3), 4.93 (1H, d, J=12.1 Hz, H-5), 2.75 (1H, dq, J=7.1, 7.3 Hz, H-11), 1.79 (<50% of [3H], s, H-15), 1.24 (3H, d, J=7.3 Hz, H-13), 0.88 (3H, d, J=6.3 Hz, H-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.78 (s).

4.2.16. [15-C^{2}H$_3$]-α-epoxy-dihydroartemisinic acid (24b)/α-epoxy-dihydroartemisinic acid (24). Oil (0.1 mg; R_t 39–43 min in the preliminary HPLC; R_t 23.3 min, 20% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 2.63 (1H, s, H-5), 2.61 (1H, dq, J=10.5, 6.9 Hz, H-11), 2.04 (1H, br m, H-6), 1.84 (1H, d, J=14.4 Hz, H-3), 1.32 (<30% of [3H], s, H-15), 1.29 (3H, d, J=6.9 Hz, H-13), 0.86 (3H, d, J=6.6 Hz, H-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.31 (s); HREIMS m/z (rel. int.): 237.1812 [M^+-H_2O, $C_{15}H_{19}{}^2H_3O_2$ requires 237.1808] (40)/234.1602 [M^+-H_2O, $C_{15}H_{22}O_2$ requires 234.1620] (12), 182 (100).

4.2.17. [15-C^{2}H$_3$]-Dihydro-*epi*-arteannuin B (25b)/dihydro-*epi*-arteannuin B (25). Oil (0.1 mg; R_t 13.8 min in the preliminary HPLC; R_t 27.6 min, 5% EtOAc/*n*-hexane). ^{1}H NMR (δ, $CDCl_3$) ppm: 3.24 (1H, dq, J=7.0, 7.1 Hz, H-11), 3.01 (1H, s, H-5), 2.42 (1H, ddd, J=10.2, 7.0, 6.6 Hz, H-7), 1.91 (1H, m, H-3), 1.88 (1H, m, H-3) 1.86–1.72 (1H, m, H-8) 1.33 (<30% of [3H], s, H-15), 1.18 (3H, d, J=7.1 Hz, H-13), 1.06 (1H, dddd, J=12.8, 12.8, 9.8, 2.0 Hz, H-9) 0.89 (3H, d, J=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 179.3 (C-12, not seen due to low intensity), 84.6 (C-6, not seen due to low intensity), 63.1 (C-4, not seen due to low intensity), 60.5 (C-5), 40.7 (C-7), 39.7 (C-1), 39.1 (C-11), 31.8 (C-9), 29.5 (C-10), 27.2 (C-3), 24.2 (C-15, not seen, <20% intensity of the other peaks), 23.5 (C-8), 20.1 (C-2 and C-14), 9.4 (C-13); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.32 (s); HREIMS m/z (rel. int.): 253.1578 [M^+, $C_{15}H_{19}{}^2H_3O_3$ requires 253.1757] (4)/250.1562 [M^+, $C_{15}H_{22}O_3$ requires 250.1569] (1), 235 (4), 232 (1), 179 (100).

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