Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron
Tetrahedron 63 (2007) 9548–9566

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

Geoffrey D. Brown[a,*] and Lai-King Sy[b]

[a]*School of Chemistry, The University of Reading, Whiteknights Road, Reading RG6 6AD, UK*
[b]*Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, Area of Excellence Scheme of University Grant Committee (Hong Kong), The University of Hong Kong, Pokfulam Road, Hong Kong, People's Republic of China*

Received 29 January 2007; revised 1 June 2007; accepted 21 June 2007
Available online 27 June 2007

Abstract—Artemisinic acid labeled with both ^{13}C and ^{2}H at the 15-position has been fed to intact plants of *Artemisia annua* via the cut stem, and its in vivo transformations studied by 1D- and 2D-NMR spectroscopy. Seven labeled metabolites have been isolated, all of which are known as natural products from this species. The transformations of artemisinic acid—as observed both for a group of plants, which was kept alive by hydroponic administration of water and for a group, which was allowed to die by desiccation—closely paralleled those, which have been recently described for its 11,13-dihydro analog, dihydroartemisinic acid. It seems likely therefore that similar mechanisms, involving spontaneous autoxidation of the $\Delta^{4,5}$ double bond in both artemisinic acid and dihydroartemisinic acid and subsequent rearrangements of the resultant allylic hydroperoxides, may be involved in the biological transformations, which are undergone by both compounds. All of the sesquiterpene metabolites, which were obtained from in vivo transformations of artemisinic acid retained their unsaturation at the 11,13-position, and there was no evidence for conversion into any 11,13-dihydro metabolite, including artemisinin, the antimalarial drug, which is produced by *A. annua*. This observation led to the proposal of a unified biosynthetic scheme, which accounts for the biogenesis of many of the amorphane and cadinane sesquiterpenes that have been isolated as natural products from *A. annua*. In this scheme, there is a bifurcation in the biosynthetic pathway starting from amorpha-4,11-diene leading to either artemisinic acid or dihydroartemisinic acid; these two committed precursors are then, respectively, the parents for the two large families of highly oxygenated 11,13-dehydro and 11,13-dihydro sesquiterpene metabolites, which are known from this species.

1. Introduction

The Chinese plant *Artemisia annua* L.[1] (sweet wormwood; Compositae) has been the subject of extensive phytochemical investigation, following the discovery more than two decades ago of the amorphane sesquiterpene, artemisinin (qinghaosu (**1**)),[2] which has now become the lead compound for an important new class of antimalarial drugs.[3] Of the 47 amorphane and cadinane sesquiterpenes, which are currently known from this species,[2,4–8] 13 incorporate a terminal double bond at the 11,13-position (Fig. 1). The most abundant sesquiterpenes from *A. annua*, which present this structural feature are artemisinic acid (qinghao acid, arteannuic acid) (**2**)[2,9–11] and arteannuin B (qinghaosu II) (**3**),[2,11,12] both of which have been suggested as biogenetic precursors to artemisinin (**1**) (see below). Other 11,13-dehydro natural products, which have been isolated in smaller amounts include: artemisitene (**4**)[13–15] (the 11,13-dehydro analog of artemisinin (**1**)), arteannuin E (qinghaosu V) (**5**),[2] arteannuin F (artemisilactone) (**6**),[16] annulide (**7**),[6,17] isoannulide (**8**),[6,17] *epi*-deoxyarteannuin B (**9**),[6,18,19] deoxyarteannuin B (**10**),[6] α-epoxyartemisinic acid (**11**),[7,20] 6,7-dehydroartemisinic acid (**12**),[15,19] a *seco*-cadinane (**13**),[21] and the methyl ester of artemisinic acid (**14**).[5]

The first ever experimental study of the biosynthesis of artemisinin (**1**), using a radioisotopically-labeled precursor, which was supplied to a plant homogenate assay system, seemed to indicate that artemisinic acid (**2**) was converted to both artemisinin (**1**) and arteannuin B (**3**) via separate pathways.[22] Subsequent investigations, using similar cell-free systems appeared to confirm this result (arrows in Scheme 1 labeled 'Refs. 22–25').[23–25] The most detail of these studies by Wang et al.[25] presented evidence that the conversion of artemisinic acid (**2**) to artemisinin (**1**) might proceed by a pathway, which involved artemisitene (**4**) as an intermediate; and that its conversion to arteannuin B (**3**) may have proceeded via α-epoxy-artemisinic acid (**11**). However, in an accompanying communication,[26] these same authors also discussed the alternative possibility that *epi*-deoxyarteannuin B (**9**)

Keywords: Terpenes and terpenoids; Biogenesis; Autoxidation; NMR.
* Corresponding author. Tel.: +44 118 378 7418; fax: +44 118 931 6331; e-mail: g.d.brown@reading.ac.uk

0040–4020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2007.06.062

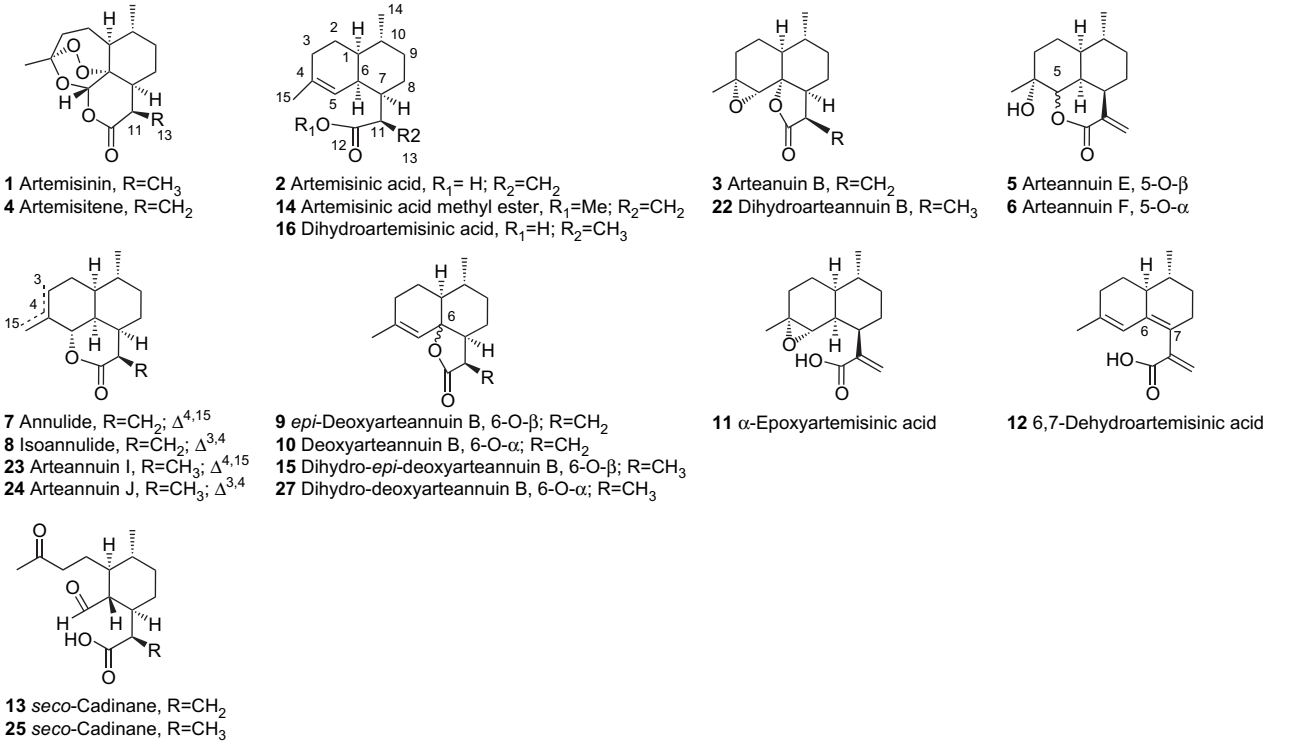
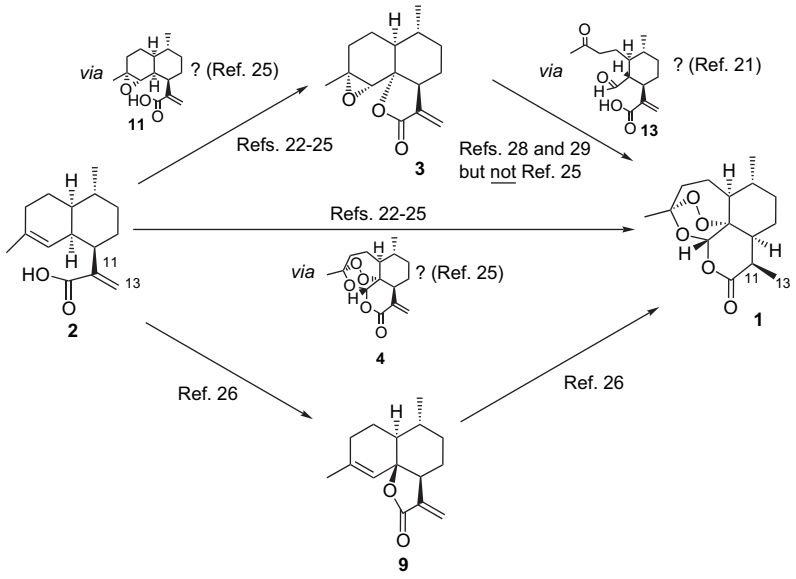


Figure 1. Amorphane and cadinane sesquiterpenes incorporating a terminal $\Delta^{11,13}$ double bond that have been reported in the literature for *A. anuua*.



Scheme 1. Various metabolic routes postulated for the biogenesis of artemisinin (**1**) from artemisinic acid (**2**) in the early literature (pre-1993), describing the biosynthesis of *A. annua*.

might be the biogenetic precursor of **1** (arrows in Scheme 1 labeled 'Ref. 26').[†] Confusingly, experimental studies by Nair and Basile,[28,29] published at around the same time, suggested that arteannuin B (**3**) could itself be a precursor to artemisinin (**1**) (indeed, a hypothetical mechanism for this transformation, involving the natural product **13**, has since been proposed).[21] Although the results of Nair and Basile supported a previous suggestion by Akhila et al.,[30] it was contradicted by Wang et al.[25] who had found that arteannuin B was *not* a precursor to artemisinin.

The various possibilities, which are presented in the early literature (pre-1993) describing the biogenesis of artemisinin are summarized in Scheme 1. Although a singular emphasis

[†] The 11,13-dihydro analog of compound **9**, dihydro-*epi*-deoxyarteannuin B (**15**) (see Fig. 1), was also suggested to be a biogenetic precursor to **1** in this reference—the companion paper[27] to this manuscript describes biosynthetic experiments, employing compound **15** as a labeled precursor, which failed to find any evidence for this proposal.

has been given to artemisinic acid (**2**) as the biosynthetic precursor of artemisinin (**1**) in this Scheme, it should be noted that additional possibilities were also considered by the original investigators. For example, the paper by Wang et al.,[25] which had described artemisinic acid (**2**) as a precursor to artemisinin (**1**), had indicated that dihydroartemisinic acid (**16**), the 11,13-dihydro analog of artemisinic acid (**2**) (see Fig. 1), was *also* a biogenetic precursor to artemisinin (**1**). The conversion of **16** to **1** obviously requires extensive oxidation to the amorphane ring system in order to generate the 1,2,4-trioxane ring of artemisinin. It should be noted that the corresponding conversion of any of the compounds **2**, **3** or **9**—all of which are proposed as biosynthetic intermediates to **1** in Scheme 1—would require a similar complex series of oxidative transformations and, *in addition*, the reduction of the $\Delta^{11,13}$ double bond at some late stage in the biogenetic route. However, there is still no experimental evidence in the literature to support such a reductive step for any of the compounds **2**, **3** or **9**.

The current study sets out to resolve the apparent confusion in the earlier literature relating to the status of artemisinic acid (**2**) as a biogenetic precursor, and to establish the true nature of its transformations in vivo in *A. annua*. In order to achieve this, use has been made of an assay system consisting of *A. annua* plants, which were fed with a labeled version of compound **2** via the cut stem, rather than plant homogenates or cell-free systems, as in all previous biosynthetic studies with artemisinic acid. The guiding philosophy has been that this more 'natural' approach should produce more reliable results to indicate the true fate of this precursor in vivo, as well as reducing the possibility for the introduction of experimental artifacts. Similarly, the use of a precursor labeled with a stable isotope (both ^{13}C and ^{2}H, in this case), has allowed information regarding the transformations undergone by labeled artemisinic acid (**2a**)[‡] to be obtained directly via the ^{13}C and ^{2}H chemical shifts of the metabolites of **2a**, which were recorded in their NMR spectra. The ability to identify metabolites in situ within crude plant extracts, without the need for the extensive sample manipulation, which is required during a chromatographic separation, further serves to reduce the possibilities for introducing experimental artifacts, in our opinion.

2. Results and discussion

2.1. Preparation of postulated biogenetic precursors

Having recently reported a synthesis[31] of doubly-labeled [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**16a**),[§] it was resolved to attempt to introduce the requisite 11,13-double bond in labeled artemisinic acid (**2a**) by making use of the oxidative selenation procedure, which is described in Section 2.1.1. The conversion of **16a** to **2a** proceeded in reasonable yield, producing sufficient quantities of labeled artemisinic acid to allow the feeding experiments with *A. annua* plants, which are described in Sections 2.2 and 2.3 to be performed. This successful synthesis encouraged further investigations in order to determine whether this same oxidative selenation strategy might also prove useful for producing some of the other 11,13-unsaturated natural products, which have been postulated as biosynthetic precursors in Scheme 1. Thus, in view of a recent synthesis of isotopically-labeled dihydro-*epi*-deoxyarteannuin B (**15a**),[27,32] the preparation of *epi*-deoxyarteannuin B (**9**) from dihydro-*epi*-deoxyarteannuin B (**15**) was also investigated, as is described in Section 2.1.2. An attempt to produce artemisitene (**4**) from artemisinin (**1**) by this same methodology is discussed briefly in Section 2.1.3.

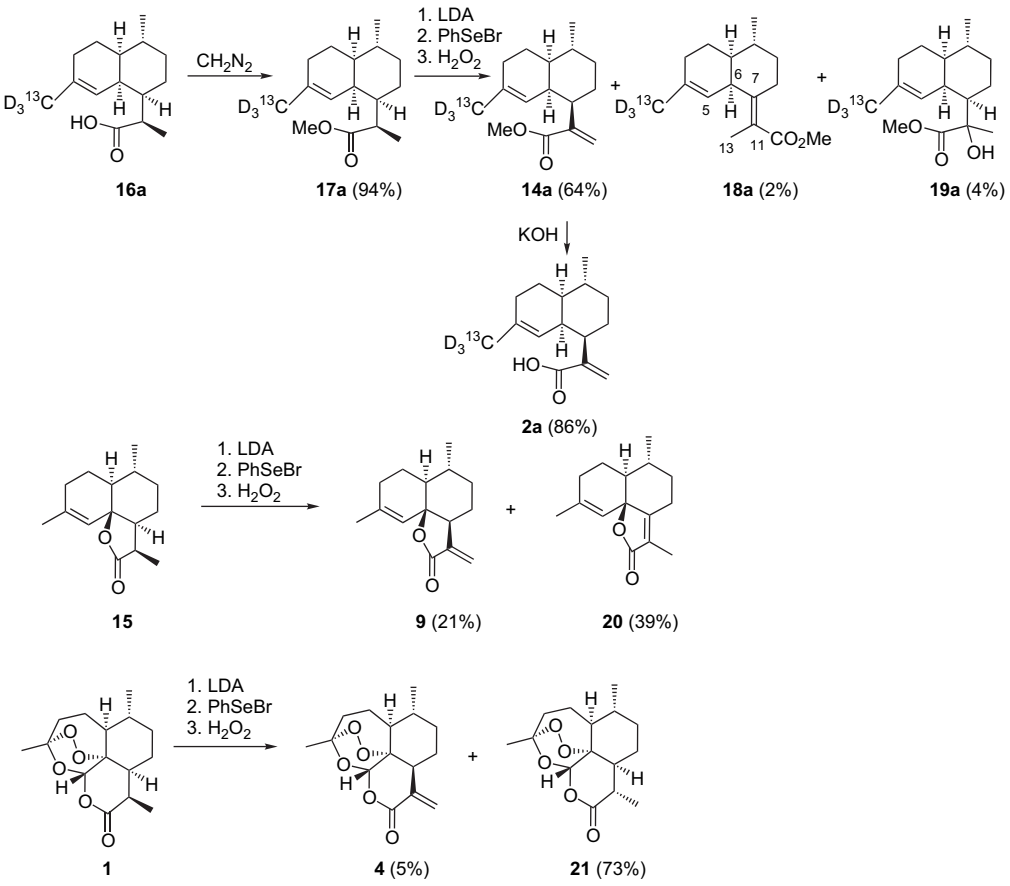
2.1.1. Preparation of [15-$^{13}C^{2}H_3$]-artemisinic acid (2a) from [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (16a). [15-$^{13}C^{2}H_3$]-Artemisinic acid (**2a**) of both high chemical and isotopic purity was prepared from [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**16a**) in three steps, as is shown in Scheme 2 (the labeled compound **16a** was itself prepared in four to five steps from commercially available artemisinin, as has been described in the literature).[31,33] Both the initial protection of labeled dihydroartemisinic acid (**16a**) as its methyl ester, compound **17a**, and the deprotection of the unsaturated methyl ester, compound **14a**, in the final step, were clean reactions, which proceeded in high yield. In contrast, the second step—an oxidative selenation reaction,[34,35] in which the $\Delta^{11,13}$ double bond required in the target compound was introduced—produced the methyl ester-protected analog of artemisinic acid **14a** in only moderate yield, together with appreciable quantities of two other isolable side-products, compounds **18a** and **19a**, which were separated chromatographically. The *E* configuration of the $\Delta^{7,11}$-double bond in the minor product **18a** was established by correlations observed in NOESY spectroscopy between H-13 and H-5/H-6.

There was no depletion of either ^{13}C or ^{2}H label in the product **2a** obtained from starting material **16a**, as judged by the results of mass spectrometry and ^{1}H, ^{2}H, and ^{13}C NMR spectroscopies, which are shown in Figure 2 (patterns for the peaks, associated with the label at the 15-position of **2a** in these spectra, present strong similarities to those, which have been previously published for the starting material, compound **16a**, in Ref. 31). The labeled product **2a** obtained from this synthesis was thus eminently suitable for use as a biosynthetic precursor in the feeding studies with *A. annua*, which are described in Sections 2.2 and 2.3. Neither labeled artemisinic acid (**2a**) nor its methyl ester derivative (**14a**) were found to undergo any detectable autoxidation[36,37] when stored in the freezer for a period of up to one year. Therefore no purification of **2a** was necessary prior to performing the feeding experiments, which are described in Sections 2.2 and 2.3. This was slightly surprising in view of the author's previous experiences with both dihydroartemisinic acid (**16**)[31] and dihdro-*epi*-deoxyarteannuin B (**15**),[27,32] which were found to have undergone appreciable autoxidation on storage, and therefore required chromatographic purification immediately prior to their use in feeding experiments.

2.1.2. Preparation of *epi*-deoxyarteannuin B (9) from dihydro-*epi*-deoxyarteannuin B (15). The efficiency of the oxidative selenation procedure[34,35] was lessened when

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

[§] It is worth stressing that each of these compounds had also been isolated in labeled form as their 11,13-dehydro analogs (compounds **2a**, **3a**, **7a**, **8a**, **9a**, and **13a**, respectively), as discussed above.



Scheme 2. Synthesis of labeled artemisinic acid (**2a**) from labeled dihydroartemisinic acid (**16a**) by an oxidative selenation procedure; and the preparation of *epi*-deoxyarteannuin B (**9**) and artemisitene (**4**) from their respective 11,13-dihydro precursors, dihydro-*epi*-deoxyarteannuin B (**15**) and artemisinin (**1**), by the same means.

introducing unsaturation to the 11,13-position of dihydro-*epi*-deoxyarteannuin B (**15**), as compared with the methyl ester of dihydroartemisinic acid (**17**), which is described in the previous section. The major product isolated from the oxidative selenation of **15** was, in fact, compound **20**, the $\Delta^{7,11}$-isomer of **9** (cf. formation of **18a** from **17a** in Section 2.1.1); while the yield of the desired product, *epi*-deoxyarteannuin B (**9**), was quite low of about 21% (Scheme 2). Although oxidative selenation could be used as a strategy for the synthesis of labeled **9**, which would be required for use in feeding experiments designed to establish whether or not **9** is a biosynthetic precursor to **1** in *A. annua* (see Scheme 1), it would seem worthwhile to first investigate alternative synthetic strategies that might give a higher yield.

2.1.3. Preparation of artemisistene (4) from artemisinin (1). Although oxidative selenation of artemisinin (**1**) did produce the desired unsaturated compound, artemisitene (**4**), again in low yield, the major product of the reaction was the saturated sesquiterpene **21**, in which the 11-methyl group of artemisinin has undergone epimerization. Oxidative selenation[38] is not therefore the preferred procedure for the synthesis of labeled **4**, which would be required for use in feeding experiments designed to establish whether or not artemisitene (**4**) is a biosynthetic precursor to **1** in *A. annua* (see Scheme 1). Rather, it would be better to convert **1** into **4** via a route, which was first reported by El-Feraly et al.,[14] involving the ene-type reaction of $^1O_2$ with an enol ether derivative of **1**. This procedure has previously been used as part of a total synthesis of 6,7-dehydroartemisinic acid (**12**),[15] and it was observed that **4** can be obtained from **1** in an overall yield of 45% by this route.

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

The presence of a carboxylic acid group in artemisinic acid allowed the aqueous solubility of this compound to be manipulated to some extent by controlling the pH of the feeding solution. As for the previous biosynthetic study with dihydroartemisinic acid,[33] it was found that the inclusion of TRIS buffer at pH 8.1 allowed sufficient [15-$^{13}C^2H_3$]-artemisinic acid (**2a**) to be incorporated reproducibly in the feeding medium (as its more soluble conjugate base form), in order that meaningful biosynthetic experiments could be performed, when using ^2H NMR spectroscopy as the primary detection method.

However, the uptake of labeled artemisinic acid (**2a**) via the root of intact plants of *A. annua* was found to be very limited by comparison with the previous studies with labeled dihydroartemisinic acid (**16a**),[33] as was shown by a preliminary experiment involving two groups of plants. In this trial, one group of plants was cut just above the root and immersed by

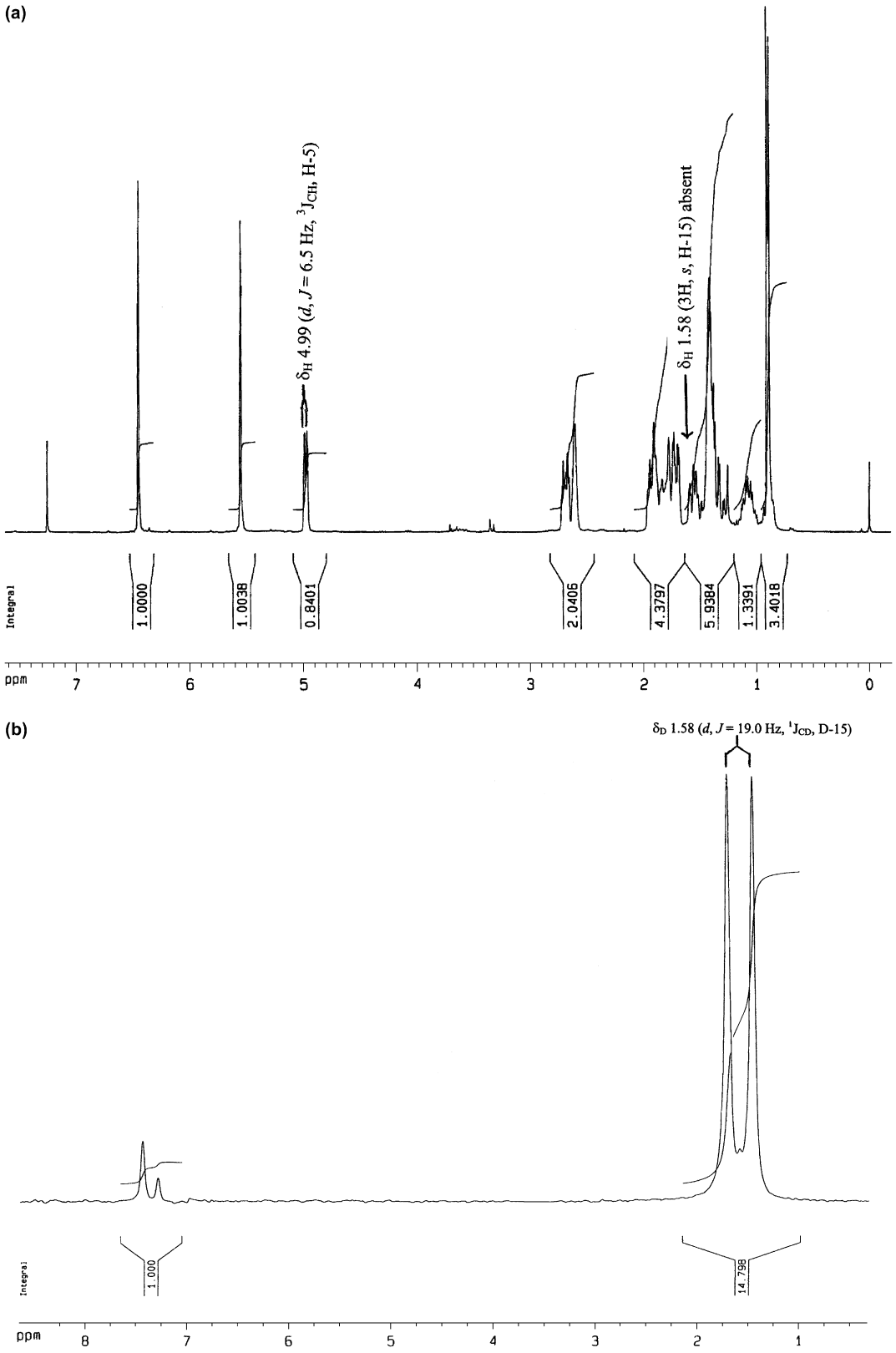


Figure 2. (a) ^{1}H NMR,[¶] (b) ^{2}H NMR, (c) ^{13}C NMR, and expansion (d) spectra of [15-$^{13}C^{2}H_3$]-artemisinic acid (**2a**) obtained by synthesis from [15-$^{13}C^{2}H_3$]-dihydroartemisinic acid (**16a**).

[¶] The region from δ_H 2.00–1.58 ppm (comprising H-2α, H-3β, H-3α, and H-9β—see Table 2) integrates as approximately [4H]; while the area from δ_H 1.58–1.20 ppm (comprising H-2β, H-1, H-10, H-8α, and H-8β—see Table 2) integrates as approximately [5H]. These integrations confirm the absence of signal for H-15, which appears as an intense three proton singlet at δ_H 1.58 ppm in the ^{1}H NMR spectrum of isotopically-normal artemisinic acid.

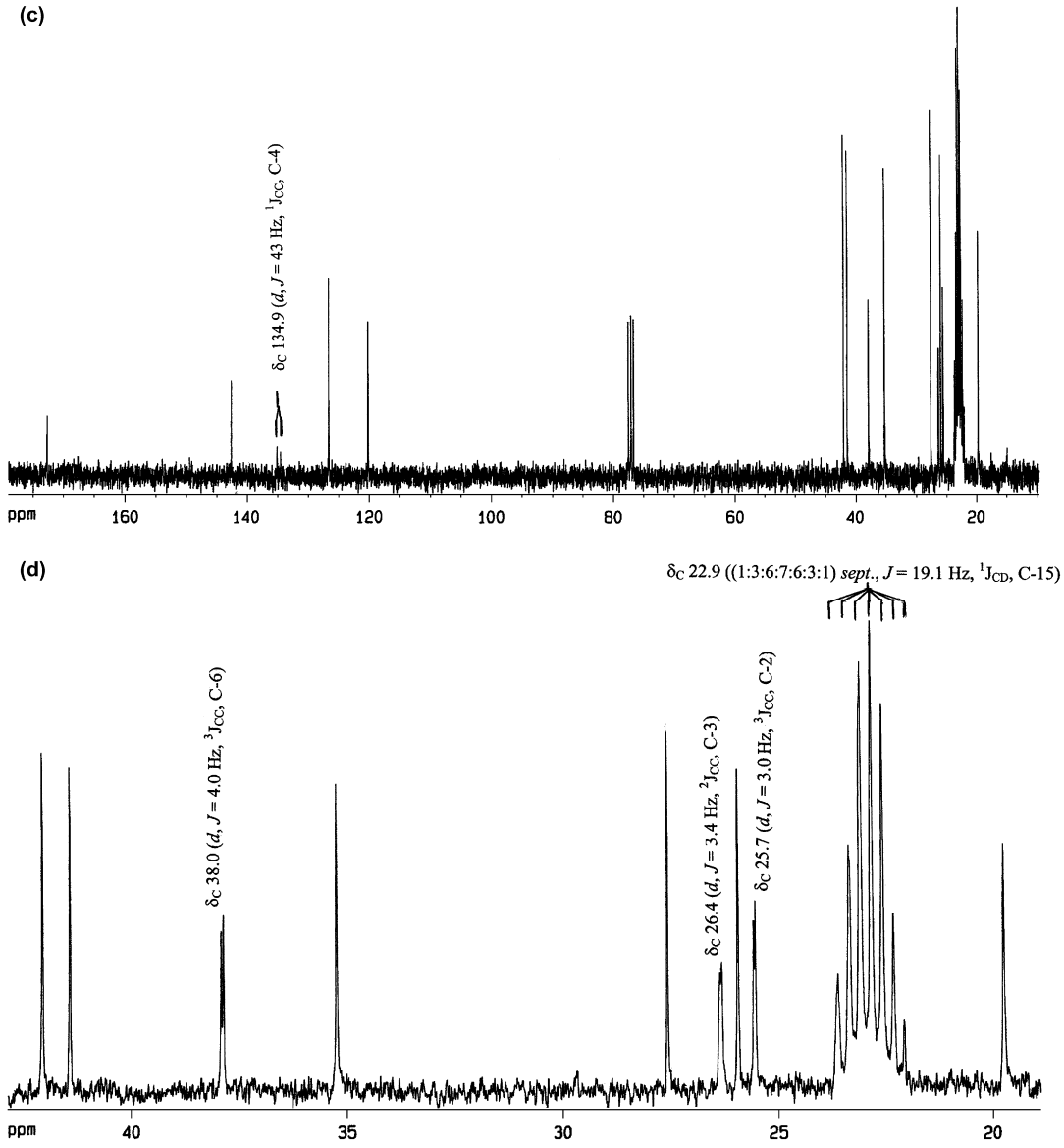


Figure 2. (*continued*)

the cut stem in a buffered feeding solution containing **2a**, while the second group, consisting of intact plants, which had been washed clean of soil, was immersed by the root. The feeding solution was taken up within 24 h by both groups (when administered at a rate of roughly 1 ml—containing ca. 1 mg of **2a**—to every 1 g of fresh plant material). Following compete uptake of the feeding solution, each group of plants was then separated into root, stem, and leaf compartments and extracted by CH_2Cl_2. The resulting crude CH_2Cl_2 extracts were immediately analyzed by ^2H NMR spectroscopy, which showed no significant metabolism over the 24-hour period of the feeding experiment (the ^2H NMR signal in each crude extract was represented predominantly by a doublet centered at δ_D 1.59 ppm, $^1J_{CD}$=19.1 Hz, corresponding to the feeding precursor, compound **2a**). However, ^2H NMR spectroscopy also indicated very significant quantitative differences in the extent of uptake of label by the two groups. Thus, while between 15 and 25% of the ^2H label was recoverable in the CH_2Cl_2 extracts from the leaf and stem compartments of the 'cut' stem-fed plants, less than 2% of the label was found in the stem compartment of the 'intact' root-fed plants, and none in the leaves. (The majority of label recovered from the 'intact' plants remained associated with the root compartment, and it is believed that this represents physical adsorption of labeled artemisinic acid on to the root surface, rather than assimilation of **2a** by the root.)

It was therefore concluded that there is a physiological barrier to the uptake of artemisinic acid by the roots of *A. annua*. This, in turn, results in a degree of uptake of artemisinic acid, which is too low to allow studies employing ^2H NMR spectroscopy as the detection method, when artemisinic acid is fed to intact plants via the root. In consequence, the feeding experiments, which are described in the next section were performed using 'stem-feeding' to cut plants. Whilst this is clearly less satisfactory than direct administration of the labeled artemisinic acid precursor to intact *A. annua* plants

via the root—as was practiced in previous experiments with dihydroartemisinic acid (for which no such physiological barrier to uptake was observed)[33]—it is still a more 'natural' feeding route than the use of homogenates or cell-free extracts, which were employed in the very first biosynthetic experiments. (The term 'natural' is used in the sense that the technique involves considerably less disturbance to the biological system, and is therefore more likely to produce results, which more closely mimic the true metabolic fate for artemisinic acid that is naturally present in *A. annua* plants.)

The recovery of ^{2}H label, which was achieved when 'stem-feeding' labeled artemisinic acid to 'cut' plants in the definitive experiments, which are described in the next section varied between 10 and 40%. Although this is quite respectable for a biosynthetic experiment, it is significantly less than the incorporations, which had been achieved in the previous experiments with dihydroartemisinic acid,[33] for which quite remarkable recoveries of up to 80% of the total administered label had been reported.

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

Forty two *A. annua* plants were cut just above the root and fed individually via the cut stem with 2 mg each of [15-$^{13}C^{2}H_3$]-artemisinic acid. These plants were then divided into two groups. One group of 21 plants was kept alive hydroponically, by periodic replacement of water in their feeding vials; while the second group was deprived of water, and allowed to die by desiccation. Three plants in each group were then harvested on a daily basis and extracted by CH_2Cl_2. The experiment was completed after 7 days when the last two sets of triplicate plant extracts had been made.

After 3 days the weight of the group of plants, which had been deprived of water was found to have dropped to about one-third of that of the group, which had been kept alive hydroponically (the weight of the live group remained relatively constant over the study). This is considered to be good evidence that this group of plants was dead well before the half-way point of the experiment was reached (or, equivalently, for the discussion that follows, that enzymatic activity would have ceased in this group of plants).

2.4. ^{2}H NMR and ^{13}C–^{2}H COSY NMR analysis of the crude CH_2Cl_2 extracts of *A. annua*, which had been fed with [15-$^{13}C^{2}H_3$]-artemisinic acid (2a)

Each CH_2Cl_2 plant extract was analyzed immediately by ^{2}H NMR spectroscopy. The extracts made after 1 day showed only a doublet peak at δ_D 1.59 ppm ($^{1}J_{CD}$=19.1 Hz), corresponding to the labeled precursor **2a**. However, this doublet peak was observed to decline over the course of the experiment, and to be replaced by several other doublets in the range δ_D 1.0–2.5 ppm from the second day onwards (weak peaks were also occasionally resolved in the range δ_D 4.8–5.3 ppm). For example, Figure 3 shows a ^{2}H NMR spectrum

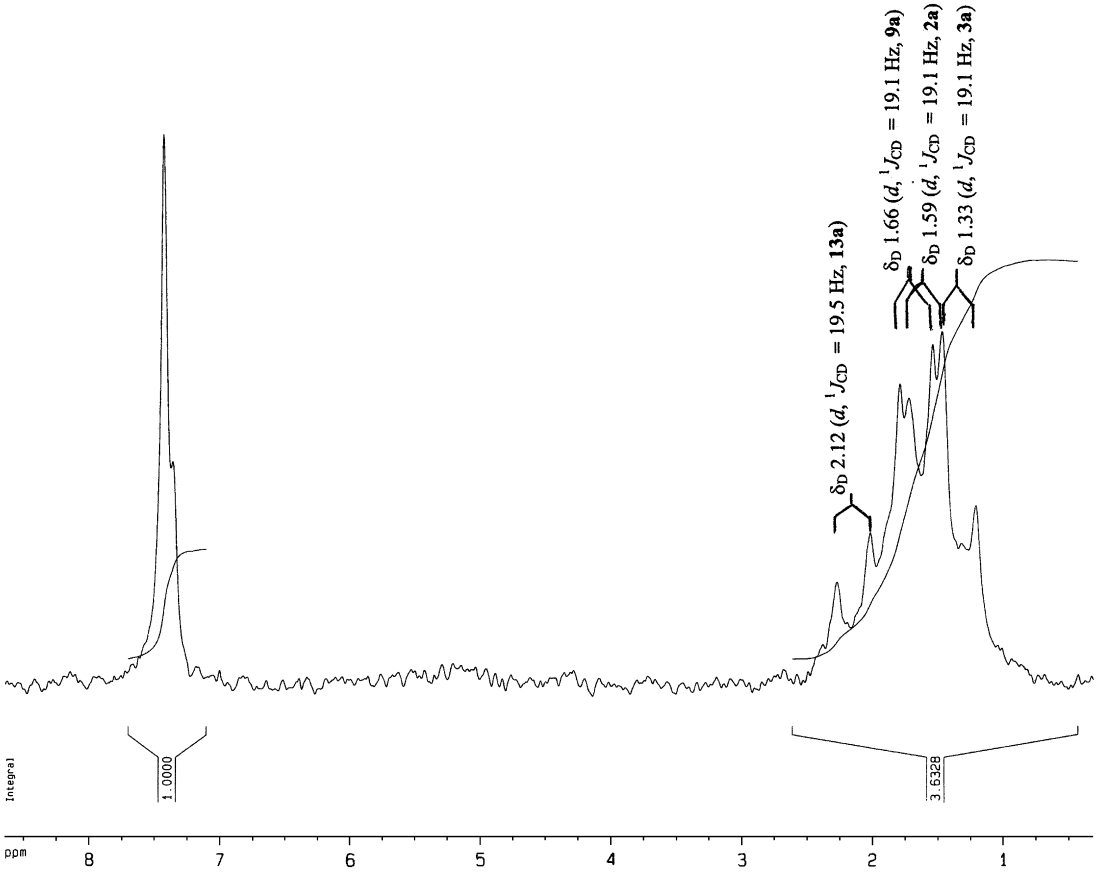


Figure 3. ^{2}H NMR spectrum of a crude CH_2Cl_2 extract from the 'dead' group of *A. annua* plants, made 4 days after feeding with [15-$^{13}C^{2}H_3$]-artemisinic acid.

from a sample, which was extracted 4 days after feeding, in which new doublet peaks at δ_D 1.33, 1.66, and 2.12 ppm have appeared, in addition to that of the labeled precursor **2a** (at δ_D 1.59 ppm).

Although the largish one-bond ^{13}C–^{2}H coupling constant ($^{1}J_{CD} \approx 19$ Hz) of these and other doubly-labeled metabolites of **2a** made a detailed visual analysis of the one-dimensional ^{2}H NMR spectra from the crude extracts of the aerial parts more difficult, it did allow for the direct analysis of these crude plant extracts by two-dimensional ^{13}C–^{2}H correlation NMR spectroscopy.[33,39,40] ^{13}C–^{2}H COSY analysis of the crude plant extracts (Fig. 4) indicated that the most abundant deuterium resonances at δ_D 1.59, 1.33, 1.66, and 2.12 ppm were connected by a single bond to carbon resonances at δ_C 22.8, 21.9, 22.3, and 29.0 ppm, respectively.

When allowance is made for the effects of ^{2}H isotopic enrichment on ^{13}C chemical shift (resulting in a ca. 0.3 ppm upfield shift in the ^{13}C resonance for each deuterium atom, which is directly attached to the carbon), the isotopically-normal values for each of these four metabolites can then be inferred as: δ_C 23.7, 22.7, 23.2, and 29.9 ppm, respectively, assuming that there has been complete retention of the ^{2}H label in the 15-methyl group. (Note that the carbon in the 15-position of isotopically-normal artemisinic acid resonates at δ_C 23.8 ppm, which is in good agreement with the carbon chemical shift—observed at δ_C 22.8 ppm for labeled **2a** in this ^{13}C–^{2}H COSY experiment—according to such an analysis). The deuterium and carbon chemical shifts recorded at the 15-position for the four metabolites, which can be identified in Figure 4 would then be entirely consistent with their assignments as compounds **2a**,[2,9–11] **3a**,[2,11,12] **9a**,[6,18,19] and **13a**,[21] respectively, based on the fully-assigned NMR data, which has been reported in the literature for each of these compounds (see references). No signals were observed in the 2D-^{13}C–^{2}H COSY spectra of any of the crude extracts that would suggest the presence of either of the labeled metabolites [15-$^{13}C^{2}H_3$]-artemisinin (**1**) or [15-$^{13}C^{2}H_3$]-artemisitene (**4**), both of which have been proposed previously as metabolites of **2** in *A. annua* (see Section 1).[22–25]

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-$^{13}C^{2}H_3$]-artemisinic acid (2a)

In order to confirm the structures of the metabolites of **2a** whose identities had been suggested in the previous section from the ^{13}C and ^{2}H NMR chemical shifts observed at their 15-position, the crude CH_2Cl_2 extracts obtained from feeding [15-$^{13}C^{2}H_3$]-artemisinic acid (**2a**) to both the 'live' and 'dead' groups of *A. annua* plants were subjected to extensive HPLC chromatographic separations (see Section 4.5). This procedure resulted in the isolation of seven doubly-labeled amorphane and cadinane sesquiterpenes (Scheme 3), in

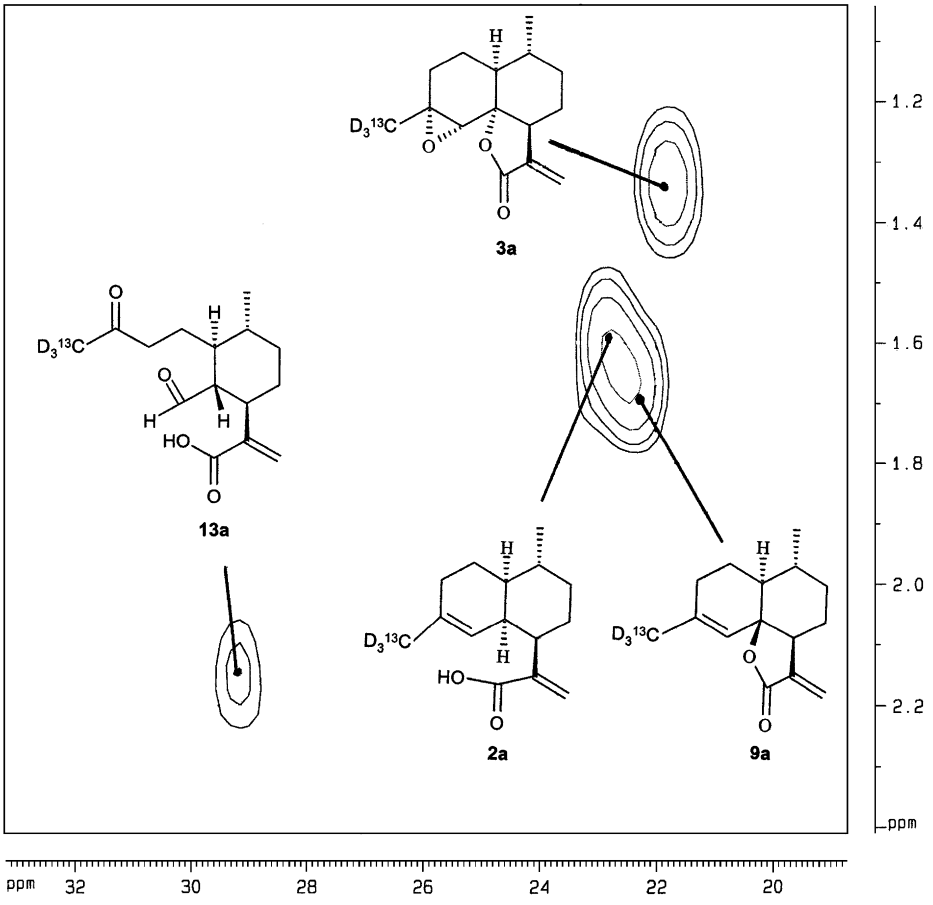
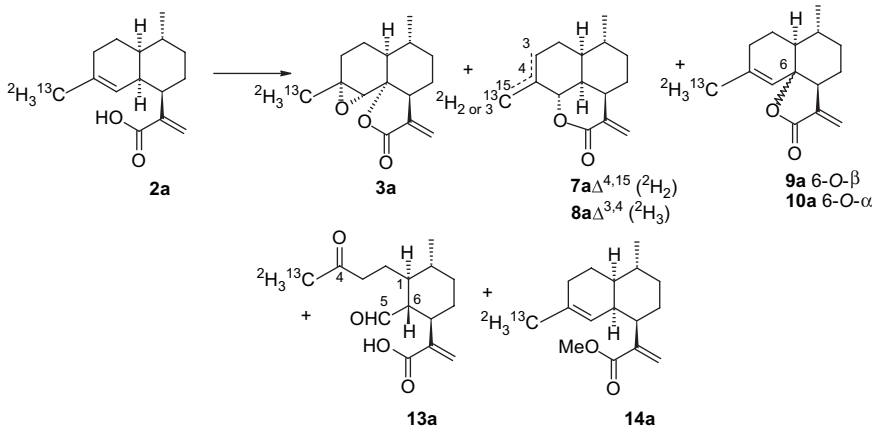


Figure 4. Expansion of the ^{13}C–^{2}H COSY spectrum from three combined CH_2Cl_2 plant extracts of *A. annua*, which had been fed with [15-$^{13}C^{2}H_3$]-artemisinic acid (**2a**), then allowed to die by desiccation, and extracted after 4 days.



Scheme 3. Labeled metabolites **3a**, **7a**, **8a**, **9a**, **10a**, **13a**, and **14a**, which were isolated following chromatographic separation of the crude plant extracts from the in vivo transformations of [15-$^{13}C^{2}H_3$]-artemisinic acid (**2a**) in *A. annua*.

addition to the recovery of the untransformed labeled feeding precursor **2a**. The structure and relative stereochemistry of the seven labeled metabolites: **3a**, **7a**, **8a**, **9a**, **10a**, **13a**, and **14a** were all then unambiguously confirmed by comparison with physical data (in particular ^{1}H and ^{13}C NMR spectra), which has been reported in the literature.

The most abundant labeled compound to be recovered from HPLC separation was the untransformed feeding precursor [15-$^{13}C^{2}H_3$]-artemisinic acid (**2a**). Analysis by ^{1}H NMR and MS of the purified sample of artemisinic acid, which was recovered after feeding suggested that it consisted of approximately 15% of the doubly-labeled form of dihydroartemisinic acid (**2a**) and ca. 85% the intensity of isotopically-normal dihydroartemisinic acid (**2**). Thus, the integral for the 15-position at δ_H 1.59 in ^{1}H NMR was ca. 85% of that expected for [3H]; and molecular ions were observed in an approximately 6:1 ratio for M^+: M+4 in mass spectrometry. Significantly, there was no evidence in either ^{13}C NMR or MS for the accumulation of any partially labeled forms of artemisinic acid, beyond those, which were already present in the sample of synthetic **2a** that had been used as the feeding precursor. Thus, while the mass spectrum of artemisinic acid, which was recovered from the feeding experiment contained both a molecular ion for isotopically-normal artemisinic acid (at m/z=234) as well as a second less abundant peak for the $^{13}C^{2}H_3$-labeled form (at m/z=238), only very low abundance peaks were observed at m/z 237 and 236, which might correspond to the partially labeled [15-$^{13}C^{2}H_2H$] and [15-$^{13}C^{2}HH_2$] forms, respectively. Similarly, the ^{13}C NMR spectrum of this sample consisted predominantly of a singlet at δ_C 23.7 ppm (for C-15 of isotopically-normal artemisinic acid) as well as a 1:3:6:7:6:3:1 septet at δ_C 22.8 ppm (ca. 0.9 ppm upfield of the isotopically-normal value, due to the presence of a $^{13}C^{2}H_3$ label at the 15-position of **2a**). The difficulties encountered in definitively identifying either a 1:2:3:2:1 quintet at ca. 0.6 ppm upfield of the isotopically-normal value, which would correspond to the partially labeled [15-$^{13}C^{2}H_2H$] form of **2a**, or a 1:1:1 triplet at ca. 0.3 ppm upfield of the isotopically-normal value, which would correspond to the partially labeled [15-$^{13}C^{2}HH_2$] form of **2a**, confirmed the low abundance of these partially deuteriated forms. It seems therefore that the isotopically-normal form of artemisinic acid, which was recovered from chromatography represents the endogenous natural product, which would have been present in *A. annua* in any case, even if labeled artemisinic acid had not been administered to the plant. Hence, it can be concluded that there has been no depletion of isotopic label as a result of metabolism. This interpretation was further confirmed by ^{1}H NMR, ^{13}C NMR, and MS analyses of the seven purified labeled metabolites: **3a**, **7a**, **8a**, **9a**, **10a**, **13a**, and **14a**, which were also isolated by chromatography (see Sections 4.5.2–4.5.8). Each of these samples exhibited a comparable ratio of the endogenous isotopically-normal form to the labeled form of the natural product (the labeled secondary metabolite consistently accounted for between 10 and 30% of the total), with no significant quantities of partially labeled forms being observed.

The most abundant metabolite of **2a** to be isolated from HPLC separation was [15-$^{13}C^{2}H_3$]-arteannuin B (**3a**). Arteannuin B has been implicated previously as a metabolite of artemisinic acid[22–25] and was also one of the first natural products ever to have been isolated from *A. annua*.[2] The next most significant metabolites were *epi*-deoxyarteannuin B (**9a**)[25] and the *seco*-cadinane (**13a**),[21] both of which have also been suggested previously as metabolites of artemisinic acid (Scheme 1), although this is the first experimental evidence that **2** can indeed be transformed into **13**. Doublet peaks for each of **3a**, **9a**, and **13a**, are clearly visible in the ^{2}H NMR spectrum of the crude CH_2Cl_2 extract, which is shown in Figure 3 (at δ_D 1.33, 1.66, and 2.12 ppm, respectively), as well as in the ^{13}C–^{2}H COSY spectrum of Figure 4 (at δ_D 1.33/δ_C 21.9, δ_D 1.66/δ_C 22.3, and δ_D 2.12/δ_C 29.0 ppm, respectively), confirming that the isolation of these compounds is unlikely to have been an artifact of the chromatographic procedures. The remaining four metabolites of labeled artemisinic acid: [15-$^{13}C^{2}H_2$]-annulide (**7a**), [15-$^{13}C^{2}H_3$]-isoannulide (**8a**), [15-$^{13}C^{2}H_3$]-deoxyarteannuin B (**10a**), and [15-$^{13}C^{2}H_3$]-artemisinic acid methyl ester (**14a**) were all isolated in trace amounts. All four metabolites have also been reported previously as *bona fide* natural products from *A. annua* (see Section 1).

Although a large number of other natural products were also isolated from this chromatographic separation (including several non-sesquiterpenoid metabolites that are typical of

A. annua), none contained a detectable amount of ^{2}H label. It was of particular interest to establish the presence or absence of label in all of those 11,13-dihydro sesquiterpenes, which were also isolated in pure form by this chromatographic separation. These included dihydroartemisinic acid (**16**), dihydroarteannuin B (**22**), arteannuin I (**23**), arteannuin J (**24**), dihydro-*epi*-deoxyarteannuin B (**15**), and the *seco*-cadinane **25** (Fig. 1).[§] In addition, pure samples of the 11,13-dihydro metabolites: arteannuins H, K, L, and M,[4] deoxyartemisinin, and artemisinin (**1**) were also isolated (however, the 11,13-dehydro analogs of these compounds were not obtained from chromatography). ^{2}H NMR spectra of each of these purified 11,13-dihydro metabolites showed no detectable ^{2}H signal, even when acquired for extended periods of time. In particular, the ^{2}H NMR spectrum of a ca. 100 mg sample of pure artemisinin, which was recovered from the feeding experiment contained no detectable deuterium signal, even when recorded with 15,000 repetitions of the pulse sequence (for comparison, experiments employing between 10 and 100 transients were generally more than sufficient to identify a ^{2}H-labeled metabolite in a chromatographic fraction, when purifying a labeled metabolite, which was present in that fraction at a level of 0.1 mg or more). The apparent absence of any ^{2}H label from all of the 11,13-dihydro metabolites, which were isolated in this study is discussed further in the next section.

3. Conclusion

The in vivo transformations of artemisinic acid, which have been observed in *A. annua* in this study are summarized in Scheme 3. The first and most obvious conclusion to be drawn from this feeding experiment is that arteannuin B (**3**) is the major metabolite from the in vivo transformations of artemisinic acid (**2**) in *A. annua* plants (Scheme 3). This is in agreement with studies in the earlier literature,[22–25] which were performed with radioisotopic labels and plant homogenates (see arrow labeled 'Refs. 22–25' in Scheme 1). A further six stable isotope-labeled metabolites were also recorded from the current study (Scheme 3), in addition to labeled arteannuin B (**3a**). One of these compounds, *epi*-deoxyarteannuin B (**9**), has previously been suggested to be an intermediate in the metabolism of artemisinic acid *en route* to artemisinin[26] (arrow labeled 'Ref. 26' in Scheme 1); but the remaining five metabolites (**7**, **8**, **10**, **13**, and **14**) have not been reported previously as metabolites of artemisininic acid (**2**), although all are known as natural products from *A. annua* (Fig. 1).

Perhaps the most intriguing result from this study is the absence of any detectable incorporation of label into artemisinin (**1**) itself. (Indeed, artemisitene (**4**), the 11,13-dihydro derivative of artemisinin, which might also have been expected to incorporate ^{2}H isotopic label, based on the earlier biosynthetic studies[25] that are summarized in Scheme 1, was not even detected in these experiments.)[‖] It can be concluded therefore that artemisinic acid (**2**) is not being converted directly into artemisinin (**1**) to any significant extent by the 'direct' route, which is indicated by the arrow labeled 'Refs. 22–25' in Scheme 1. Two suggestions can be made to explain the apparent anomaly between earlier studies—conducted with cell-free systems and radioisotopes[22–26,28–30]—which appear to have observed the direct conversion of artemisinic acid (**2**) to artemisinin (**1**), and the current study—conducted in vivo with a stable isotope label—which does not confirm this. Firstly, it may be that the cell-free systems, which were employed in the earlier biosynthetic studies[22–26,28–30] were inappropriate, permitting the appearance of artifacts, which have not been observed in the current experiment, when stem-feeding the *A. annua* plants (indeed, several recent papers have made reference to the possibility of artifacts arising from the autoxidation of various natural products from *A. anuua*).[31–33,36,37,39,40] Secondly, it is well known that the use of a radioisotopic label can permit the detection of a metabolite at a level, which is typically several orders of magnitude lower than that for a stable isotope label. Hence, if a *bona fide minor* biosynthetic pathway from artemisinic acid to artemisinin does exist, then it is quite possible that this transformation would have been observed when using radioisotopes, but would have escaped detection with stable isotopes. (Or, alternatively, this result could also be explained if artemisinic acid was to be degraded to simpler metabolites, which were then capable of re-assembly to artemisinin by the cell-free assay system, if even to only a very limited extent).

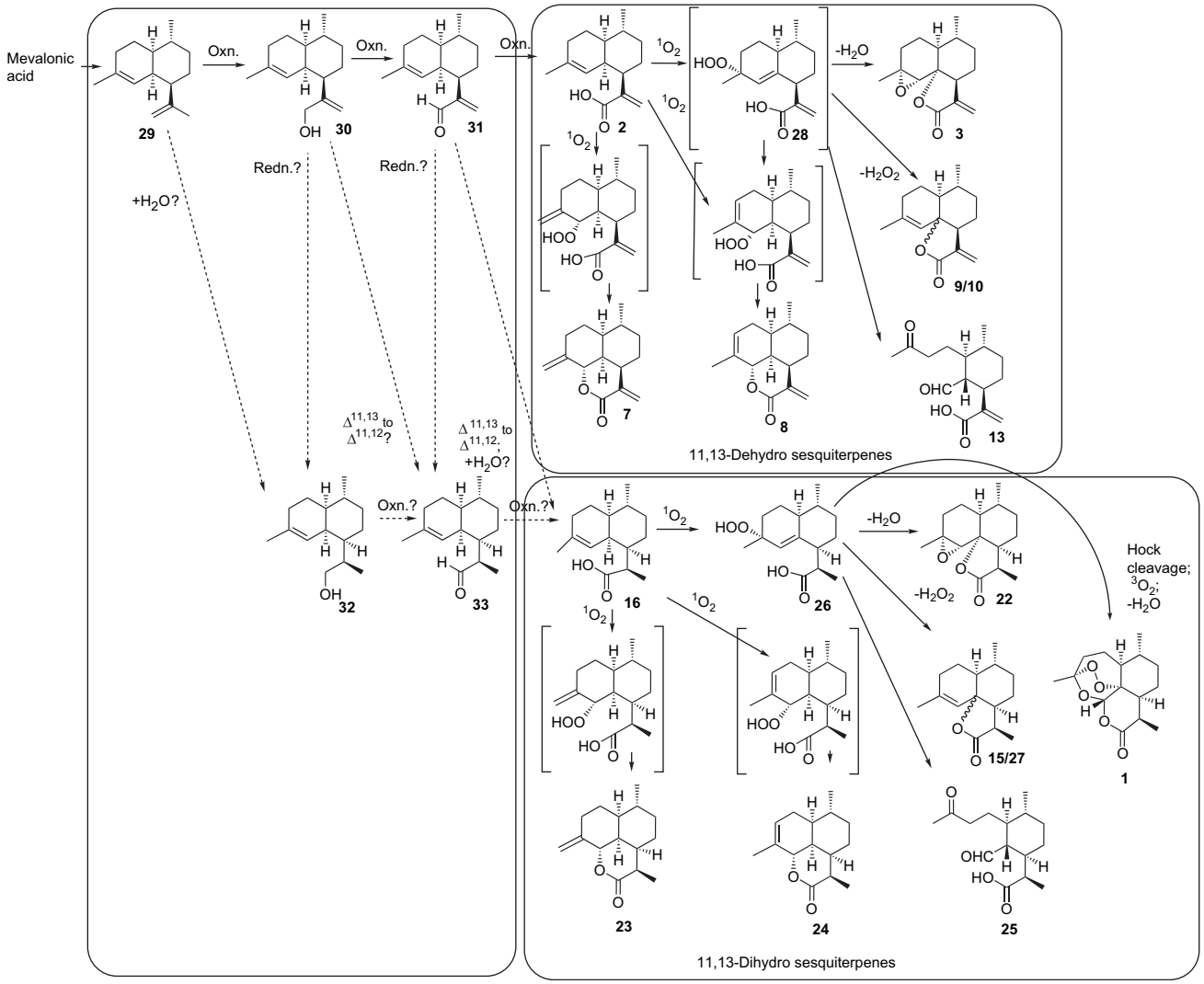
Although these results appear to rule out any significant role for the 'direct' pathway from artemisinic acid (**2**) to artemisinin (**1**), which is shown in Scheme 1, it is still possible that the 'indirect' route, which involves arteannuin B (**3**) as an intermediate (upper arrow from **2** to **3**, labeled 'Refs. 22–25'; then arrow from **3** to **1**, labeled by 'Refs. 28 and 29 but *not* Ref. 25'), may play a role in the biogenesis of **1**. The current feeding experiment was terminated after 7 days, and it is conceivable that—if the rate of conversion of arteannuin B (**3**) to artemisinin (**1**) was very much slower than the experimentally-observed rate of conversion of artemisinic acid (**2**) to arteannuin B (**3**)—there would have been insufficient time for a detectable amount of labeled artemisinin to be accumulated by this route. This issue could most easily be resolved by performing a future feeding experiment using stable isotope-labeled arteannuin B (**3a**) as a hypothetical biogenetic precursor.[22–25] Similarly, it is also possible that either *epi*-deoxyarteannuin B (**9**)[26] or the *seco*-cadinane **13**[21] (both of which were isolated in significantly smaller amounts than **3**), might be undergoing conversion to artemisinin at a rate, which is much slower than the rate of their formation from artemisinic acid (**2**). For this reason, it might also be worthwhile performing future feeding experiments using either stable isotope-labeled *epi*-deoxyarteannuin B (**9a**) or labeled *seco*-cadinane (**13a**) as the hypothetical biogenetic precursor.

Although the absence for any detectable transformation of **2** into **1** in this study is at variance with the earlier literature (pre-1993)[22–26,28–30] for the biosynthesis of artemisinin, it is consistent with the more recent literature (post-1999),[33,41–44] in which there is now a gathering consensus that dihydroartemisinic acid (**16**), rather than artemisinic

[‖] However, this is not too surprising, since artemisitene is a 'scarce' natural product, which has been reported only very infrequently from nature.[13] The author has never isolated artemisitene from *A. annua*, although he has reported some of the most extensive and detailed phytochemical investigations of this species to date.[4–7]

acid (**2**), is the true late-stage precursor to artemisinin. In this regard, it is intriguing to note that there is a very high degree of structural homology between the various highly oxygenated 11,13-dehydro sesquiterpenes, which have been isolated as metabolites of artemisinic acid (**2**) in the current study, and those 11,13-dihydro metabolites, which were obtained in the previous study with dihydroartemisinic acid (**16**).[33] In the previous study, the complex pattern of metabolism observed for **16** was concisely explained in terms of the 'ene'-type addition of singlet oxygen to the $\Delta^{4,5}$-double bond of dihydroartemisinic acid. This then led to a detectable tertiary allylic hydroperoxide intermediate (**26**), which underwent reactions characteristic of such allylic hydroperoxides to produce a variety of compounds, including **15**, **22**–**25**, and **27**—as well as artemisinin (**1**) itself—as is shown in the bottom right hand box of Scheme 4. Although the putative tertiary allylic hydroperoxide of artemisinic acid, compound **28** (i.e., the 11,13-dehydro analog of intermediate **26**), was not isolated as a metabolite in the current study of the metabolism of artemisinic acid (**2**), its presence has been inferred in the top right hand box of Scheme 4 (where it appears in parentheses), based on the near identical product distribution observed between the two studies (the 11,13-dihydro analogs of each of the six labeled compounds **3**, **7**, **8**, **9**, **10**, and **13** were all isolated in the previous study).[33] In support of the mechanisms for the transformations of **2**, which are proposed in the top right hand box of Scheme 4, it should be noted that the allylic hydroperoxide **28** can be produced in the laboratory by chemical reaction of **2** with $^1O_2$,[45–47] and that it has then been shown in vitro to undergo several of the transformations, which are depicted in this box. The proposal that the 'metabolism' of artemisinic acid (**2**) in *A. annua* might proceed via an allylic hydroperoxide intermediate such as compound **28**, perhaps created spontaneously by a pigment-photosensitized autoxidation reaction occurring in vivo, was further supported by the identical pattern of transformations, which were observed when feeding artemisinic acid to both the 'live' and the 'dead' groups of plants in this experiment.

The third important observation is that all of the seven metabolites isolated from this feeding experiment (Scheme 3) retained the terminal double bond, which was present at the 11,13-position of artemisinic acid (**2**). In fact, about



Scheme 4. Unified biosynthetic scheme to account for the biogenesis of the two families of 11,13-unsaturated and 11,13-saturated metabolites from *A. annua*.

two thirds of all the 11,13-dehydro sesquiterpenes, which are currently known from *A. annua* (see Fig. 1) have been isolated in this study. In contrast, none of the 11,13-saturated sesquiterpenes, which were also recovered from the crude extracts showed any detectable incorporation of label, including artemisinin (**1**) itself, as already noted. These conclusions are the exact reverse of those from the previous experiment,[33] when feeding dihydroartemisinic acid (**16**) to *A. annua*, in which about half** of all the 11,13-saturated sesquiterpene metabolites ever described from this species were isolated in labeled form, but none of the 11,13-dehydro sesquiterpenes.

The inescapable conclusion seems to be therefore that neither oxidation nor reduction at the 11,13-position can occur to any significant extent for 'advanced' metabolites such as artemisinic acid (**2**) or dihydroartemisinic acid (**16**). Artemisinic acid (**2**) is thus the exclusive precursor to the 11,13-dehydro metabolites from *A. annua*, just as dihydroartemisinic acid (**16**) is the exclusive precursor to the large family of highly oxygenated 11,13-dihydro sesquiterpenes from this species. This conclusion is summarized diagrammatically in the top and bottom right hand boxes of Scheme 4 in which artemisinic acid and dihydroartemisinic acid are portrayed as the committed intermediates on two separate pathways to the two large families of oxygenated 11,13-dehydro and 11,13-dihydro sesquiterpene natural products, which are known from *A. annua*.

Consequently, the introduction of saturation/unsaturation at the 11,13-position must be occurring at an earlier stage in the biosynthetic pathway to the sesquiterpene metabolites found in *A. annua*. In this regard, it is now well established that the very first steps in the biogenesis of sesquiterpenes from *A. annua* proceed by the normal pathways of terpenoid biosynthesis, generating amorpha-4,11-diene (**29**) as the first committed intermediate on the pathway to artemisinin (**1**).[48–50] Therefore, the oxidative/reductive interconversions, which link the separate routes to the two large families of oxygenated 11,13-dehydro and 11,13-dihydro sesquiterpenes, must be occurring at some point 'downstream' of amorpha-4,11-diene (**29**), but 'upstream' of artemisinic acid (**2**)/dihydroartemisinic acid (**16**). The left hand box in Scheme 4 summarizes recent experimental work on the metabolism of *A. annua*,[43,44,51,52] which has suggested artemisinic alcohol (**30**), artemisinic aldehyde (**31**), dihydroartemisinic alcohol (**32**) and dihydroartemisinic aldehyde (**33**) as possible metabolites of amorpha-4,11-diene (**29**). Intriguingly, a single cytochrome P_{450}[44,52] has now been found, which catalyses each of the three oxidations on the route **29**→**30**→**31**→**2**, although the point at which the bifurcation to the lower path, leading to dihydroartemisinic acid (**16**) and the 11,13-dihydro series of sesquiterpenes remains unclear (possible branching points are indicated by arrows bearing question marks in the left hand box of Scheme 4). Finally, it should be noted that the unified biosynthetic pathway shown in Scheme 4 could neatly explain the oft-reported incidence of two chemotypes of *A. annua*:[53] viz.—a low-yielding-artemisinin chemotype, which is rich in artemisinic acid (i.e., biosynthesis is proceeding largely along the upper route), and a high-yielding-artemisinin chemotype, which is low in artemisinic acid (i.e., biosynthesis is proceeding predominantly by the lower route).

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 (δ) 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 this section 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 (δ_D 7.43 ppm) and as an 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 **2a** and other metabolites, which were resolved in the crude plant extract, was routinely used to estimate the amount of ^{2}H label present in such extracts).[33] The chemical shift of this reference compound did not interfere with the products of metabolism of artemisinic acid, for which ^{2}H signal was normally observed only in the aliphatic region (δ_D 1.0–2.5 ppm) or, occasionally, in the alkene region (δ_D 4.5–5.5 ppm) of the spectrum—see Section 2.4.

^{13}C–^{2}H COSY NMR[33,39,40] spectra were acquired with a Bruker AV 600 MHz NMR spectrometer, employing some modifications to the procedures, which have been described previously for a Bruker DRX 500 spectrometer.[33,39] The ^{2}H 'hard' 90° pulses (70 μs/−6 dB) and composite decoupling pulses (Waltz 16; 230 μs/8 dB), which are required for the ^{13}C–^{2}H COSY experiment were created by a '2H-TX' board (essentially a gated 20 W amplifier and interface), which was incorporated as standard on the AV 600 spectrometer. This board converted the lock channel circuitry from its normal function to a transmit/receiver mode during pulses, but allowed the normal operation of the lock at all other times—therefore samples for ^{13}C–^{2}H COSY spectroscopy could be prepared in $CDCl_3$, rather than $CHCl_3$ solution, as previously,[33,39] and it then became possible to lock and auto-shim the sample over long 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).[33,39,40]

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.

** However, note that fewer actual metabolites were detected with confidence in this experiment, as compared with the previous experiment with dihydroartemisinic acid[33] (i.e., 7 metabolites from artemisinic acid, compared with 15 from dihydroartemisinic acid). The three most critical factors in explaining this are probably: (i) the slightly smaller scale of this experiment; (ii) the lessened aqueous solubility of artemisinic acid in the feeding solution, as compared with dihydroartemisinic acid (a maximum of 1.3 mg/ml, vs 2.4 mg/ml); and (iii) the lower efficiency for the recovery of label, which was observed when feeding artemisinic acid, as compared to dihydroartemisinic acid (10–40%, as compared with 60–80%).

Table 1. ^{13}C NMR assignments (in $CDCl_3$) for some of the compounds obtained by synthesis in Section 2.1

Position (mult.)[a]	2a	14a	18a	19a	20	21
1 (CH)	41.4	41.4	41.1	42.3	47.0	50.5
2 (CH_2)	25.7	25.5	25.1	25.9	19.3	24.7
3 (CH_2)	26.4	26.3	25.6	26.2	31.6	35.9
4 (C)	134.9	134.6	135.0	134.5	143.0	105.3
5 (CH)	120.2	120.3	122.8	122.0	121.4	94.1
6 (CH)	38.0	37.9	40.4	37.0	87.3 (C)	80.7 (C)
7 (CH)	42.1	42.3	150.5 (C)	48.0	167.8 (C)	45.5
8 (CH_2)	26.0	25.9	28.7	23.6	21.5	31.1
9 (CH_2)	35.3	35.2	35.7	35.5	35.6	34.0
10 (CH)	27.6	27.5	28.2	27.7	26.3	37.6
11 (C)	142.7	143.3	120.3	77.6	122.7	39.7 (CH)
12 (C)	172.7	167.8	171.3	178.7	173.9	172.6
13 (CH_2)	126.6	124.1	15.0 (CH_3)	23.9 (CH_3)	8.6 (CH_3)	20.5 (CH_3)
14 (CH_3)	19.7	19.7	19.4	19.7	17.3	19.9
15 (CH_3)	22.9[b]	22.8[b]	22.6[b]	23.0[b]	23.2	25.5
15-OMe (CH_3)	—	51.7	51.4	52.9	—	—

[a] Multiplicity determined from DEPT.
[b] Chemical shifts shown are for the 15-$^{13}C^2H_3$ isotopomer, which is expected to be ca. 0.9 ppm upfield of the isotopically-normal value in ^{13}C NMR.

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 FTIR-8201 PC instrument. HPLC separations were performed using a Varian chromatogram equipped with RI star 9040 and UV 9050 detectors and either a YMC diol or a PREP-SIL column (both 20 mm× 25 cm), flow rate 8 mL/min.

4.1.1. Synthesis of labeled artemisinic acid (2a). Labeled artemisinic acid (**2a**, 102 mg) was obtained from labeled dihydroartemisinic acid (**16a**, 400 mg) in three steps, as described below. Ref. 31 contains details of the preparation of the starting material, compound **16a**, from artemisinin (**1**) by a reconstructive synthesis requiring four to five steps.

4.1.1.1. Methylation of [15-$^{13}C^2H_3$]-dihydroartemisinic acid (16a). Diazald® (*N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide, 0.5 g, 2.33 mmol) was dissolved in Et_2O (10 ml) and cooled in ice. A solution of KOH (0.14 g) in 95% EtOH (3 ml) was added, and the mixture was stirred at 0 °C for 10 min. The ethereal diazomethane solution was distilled from a water bath and added dropwise to a solution of dihydroartemisinic acid (400 mg, 2.27 mmol) (**16a**) in Et_2O (20 ml). When the evolution of bubbles had ceased, acetic acid (10%; 1 ml) was added to remove excess CH_2N_2. The reaction mixture was extracted by Et_2O (3×30 ml) and the combined organic extracts were washed successively with saturated $NaHCO_3$ (3×5 ml) and brine (3×10 ml), then dried ($MgSO_4$), and the organic solvent was removed under reduced pressure to yield the methyl ester **17a** (399 mg, 1.58 mmol; 94%) without the need for further purification.

*4.1.1.1.1. [15-$^{13}C^2H_3$]-Dihydroartemisinic acid methyl ester (**17a**).* Oil (see also Ref. 31 for physical properties). IR ν (cm^{-1}): 2924 (m), 2870 (m), 2849 (m), 1726 (s), 1456 (m), 1435 (m), 1215 (s). ^1H NMR (δ, $CDCl_3$) ppm: 5.12 (1H, d, J=6.7 Hz, $^3J_{CH}$, H-5), 3.67 (3H, s, 15-OMe), 2.50 (1H, m, H-6), 2.50 (1H, m, H-11), 1.94 (1H, m, H-2β), 1.91 (1H, m, H-3α), 1.80 (1H, m, H-3β), 1.62 (1H, m, H-7), 1.59 (1H, m, H-9β), 1.55 (1H, m, H-2α), 1.41 (1H, m, H-10), 1.25 (2H, m, H-1 and H-8α), 1.13 (3H, d, J=6.9 Hz, H-13), 1.08 (1H, m, H-8β), 0.94 (1H, m, H-9α), 0.86 (3H, d, J=6.5 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 177.7 C (C-12), 135.6 C (d, J=43.1 Hz, $^1J_{CC}$, C-4), 119.4 CH (C-5), 51.2 CH_3 (15-OMe), 43.9 CH (C-7), 42.0 CH (C-11), 41.7 CH (C-1), 36.3 CH (d, J=4.0 Hz, $^3J_{CC}$, C-6), 35.2 CH_2 (C-9), 27.6 CH (C-10), 27.4 CH_2 (C-8), 26.5 CH_2 (d, J=3.5 Hz, $^2J_{CC}$, C-3), 25.7 CH_2 (d, J=2.9 Hz, $^3J_{CC}$, C-2), 22.8 ((1:3:6:7:6:3:1) sept., J=19.2 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.6 CH_3 (C-14), 15.0 CH_3 (C-13); ^2H NMR (δ, $CHCl_3$) ppm: 1.62 (d, J=19.2 Hz, $^1J_{CD}$, 15-D_3); HREIMS *m/z* (rel int.): 254.2156 [M^+, $C_{15}{}^{13}C_1H_{23}{}^2H_3O_2$ requires 254.2154] (1.6), 253.2084 [M^+, $C_{15}{}^{13}C_1H_{24}{}^2H_2O_2$ requires 253.2092] (0.1), 207 (4), 205 (4), 166 (100), 153 (25), 136 (18).

4.1.1.2. Introduction of the $\Delta^{11,13}$ double bond in 14a by oxidative selenation of 17a. Dry THF (3 ml) was cooled to −78 °C under an atmosphere of N_2 and distilled diisopropylamine (0.16 ml, 1.14 mmol) and *n*-butyllithium (0.7 ml, 1.6 M in hexane, 1.12 mmol) were added with stirring over a period of 1 h. A solution of [15-$^{13}C^2H_3$]-dihydroartemisinic acid methyl ester (**17a**) (0.20 g, 0.79 mmol) in THF (1 ml) was added and stirring continued for 30 min, then distilled hexamethylphosphoramide (0.3 ml) was added and stirring continued for a further 30 min. The temperature was raised to −10 °C and the mixture stirred for another 2 h, then the reaction mixture was cooled to −78 °C prior to the transfer of a solution of phenylselenyl bromide (0.189 g, 0.80 mmol) in THF (2 ml). The reaction mixture was stirred for 15 min before the temperature was increased to −10 °C and stirring was then continued for another 1 h. Finally, acetic acid (1 ml; 25%) and H_2O_2 (0.5 ml; 30% in H_2O) were added and the mixture was stirred at room temperature for 30 min. The product was extracted with Et_2O (3×50 ml) and the combined organic layers were washed by saturated $NaHCO_3$ (3×10 ml), followed by brine (3×10 ml), then dried ($MgSO_4$), and solvent was removed in vacuo to yield a crude product (0.31 g) consisting of compounds **14a**, **18a**, and **19a**, which were separated by HPLC (diol column, *n*-hexane).

*4.1.1.2.1. [15-$^{13}C^2H_3$]-Artemisinic acid methyl ester (**14a**).* Oil (126 mg, 64%; t_R 12.3 min) (see also Ref. 5 and Section 4.5.8 for physical properties). IR ν (cm^{-1}): 3030 (w), 2951 (s), 2924 (s), 2870 (s), 1713 (s), 1624 (m), 1434 (m), 1271 (m), 1159 (s). ^1H NMR (δ, $CDCl_3$) ppm: 6.28

Table 2. ^{1}H NMR assignments (in $CDCl_3$) for compounds obtained by synthesis in Section 2.1

Position	2a	14a	18a	19a	20	21
1	1.43	1.43	1.37	1.22	1.83	1.47
2α	1.92	1.93	1.97	1.93	1.82	1.94
2β	1.53	1.53	1.54	1.59	1.82	1.49
3α	1.74	1.76	1.81	1.79	2.16	2.08
3β	1.88	1.88	1.94	1.89	2.28	2.39
5	4.99	4.98	4.96	5.76	5.40	5.93
6	2.61	2.54	3.34	2.72	—	—
7	2.67	2.72	—	1.77	—	1.66
8α	1.38	1.40	2.79	1.42	2.63	1.80
8β	1.32	1.31	1.91	1.08	2.25	1.44
9α	1.04	1.07	1.00	0.94	1.41	1.12
9β	1.70	1.71	1.68	1.66	1.86	1.71
10	1.42	1.44	1.62	1.49	1.14	1.42
11	—	—	—	—	—	2.28
13	6.45, 5.55	6.28, 5.43	1.88	1.43	1.82	1.47
14	0.89	0.90	0.89	0.86	0.84	1.00
15	1.58[a]	1.59[a]	1.62[a]	1.60[a]	1.69	1.46
15-OMe	—	3.74	3.37	3.78	—	—

[a] Not observed in the ^{1}H NMR spectrum of the [15-$^{13}C^2H_3$]-isotopomer.

(1H, dd, J=1.2, 1.0 Hz, H-13a), 5.43 (1H, dd, J=1.4, 1.2 Hz, H-13b), 4.98 (1H, d, J=6.7 Hz, $^3J_{CH}$, H-5), 3.74 (3H, s, 15-OMe), 2.72 (1H, ddd, J=12.2, 3.5, 3.5 Hz, H-7), 2.54 (1H, br, H-6), 0.89 (3H, d, J=6.1 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm: 167.8 C (C-12), 143.3 CH (C-11), 134.6 (d, J=43.3 Hz, $^1J_{CC}$, C-4), 124.1 CH_2 (C-13), 120.3 CH (C-5), 51.7 CH_3 (15-OMe), 42.3 CH (C-7), 41.4 CH (C-1), 37.9 CH (d, J=4.0 Hz, $^3J_{CC}$, C-6), 35.2 CH_2 (C-9), 27.5 CH (C-10), 26.3 CH_2 (d, J=2.9 Hz, $^2J_{CC}$, C-3), 25.9 CH_2 (C-8), 25.5 CH_2 (d, J=2.6 Hz, $^3J_{CC}$, C-2), 22.8 ((1:3:6:7:6:3:1) sept., J=19.1 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.7 CH_3 (C-14)—see also Table 1; ^{2}H NMR (δ, $CHCl_3$) ppm: 1.58 (d, J=19.1 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 252.1998 [M^+, $C_{15}{}^{13}C_1H_{21}{}^2H_3O_2$ requires 252.1998] (75), 251 (5), 250 (2), 249 (0.5), 237 (7), 220 (33), 207 (12), 192 (51), 166 (36), 153 (100).

*4.1.1.2.2. [15-$^{13}C^2H_3$]-E-7,11-Dehydro-dihydroartemisinic acid methyl ester (**18a**).* Oil (4.5 mg, 2%; t_R 13.9 min). IR ν (cm^{-1}): 3028 (m), 2924 (m), 2870 (m), 1709 (s), 1457 (m), 1435 (s), 1286 (m), 1248 (m), 1198 (s). ^{1}H NMR (δ, $CDCl_3$) ppm: 4.96 (1H, d, J=6.6 Hz, $^3J_{CH}$, H-5), 3.73 (3H, s, 15-OMe), 3.34 (1H, br s, H-6), 2.79 (1H, dddd, J=13.7, 1.6, 1.6, 1.6 Hz, H-8), 1.88 (3H, d, J=1.8 Hz, H-13), 0.89 (3H, d, J=6.6 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm: 171.3 C (C-12), 150.5 C (C-7), 135.0 C (d, J=42.9 Hz, $^1J_{CC}$, C-4), 122.8 CH (C-5), 120.3 C (C-11), 51.4 CH_3 (15-OMe), 41.1 CH (C-1), 40.4 CH (d, J=4.0 Hz, $^3J_{CC}$, C-6), 35.7 CH_2 (C-9), 28.7 CH_2 (C-8), 28.2 CH (C-10), 25.6 CH_2 (d, J=3.0 Hz, $^2J_{CC}$, C-3), 25.1 CH_2 (d, J=3.0 Hz, $^3J_{CC}$, C-2), 22.6 ((1:3:6:7:6:3:1) sept., J=19.0 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.4 CH_3 (C-14), 15.0 CH_3 (C-13)—see also Table 1; ^{2}H NMR (δ, $CHCl_3$) ppm: 1.62 (d, J=19.0 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 252.1995 [M^+, $C_{15}{}^{13}C_1H_{21}{}^2H_3O_2$ requires 252.1998] (52), 251 (5), 250 (2), 249 (0.5), 220 (38), 193 (12), 166 (50), 153 (100), 136 (72).

*4.1.1.2.3. [15-$^{13}C^2H_3$]-11-Hydroxy-dihyroartemisinic acid methyl ester (**19a**).* Oil. (7.6 mg, 4%; t_R 55 min). IR ν (cm^{-1}): 3528 (m), 2926 (m), 2870 (m), 1724 (s), 1456 (m), 1228 (s). ^{1}H NMR (δ, $CDCl_3$) ppm: 5.76 (1H, d, J=6.8 Hz, $^3J_{CH}$, H-5), 3.78 (3H, s, 15-OMe), 3.21 (1H, s, –OH), 2.72 (1H, br s, H-6), 1.43 (3H, s, H-13), 1.08 (1H, br d, J=12.9 Hz, H-8), 0.94 (1H, dddd, J=12.9, 12.7, 12.7, 3.4 Hz, H-9), 0.86 (3H, d, J=6.6 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm: 178.7 C (C-12), 134.5 C (d, J=43.2 Hz, $^1J_{CC}$, C-4), 122.0 CH (C-5), 77.6 C (C-11), 52.9 CH_3 (15-OMe), 48.0 CH (C-7), 42.3 CH (C-1), 37.0 CH (d, J=4.2 Hz, $^3J_{CC}$, C-6), 35.5 CH_2 (C-9), 27.7 CH (C-10), 26.2 CH_2 (d, J=3.1 Hz, $^2J_{CC}$, C-3), 25.9 CH_2 (d, J=2.6 Hz, $^3J_{CC}$, C-2), 23.9 CH_3 (C-13), 23.6 CH_2 (C-8), 23.0 ((1:3:6:7:6:3:1) sept., J=18.9 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.7 CH_3 (C-14)—see also Table 1; ^{2}H NMR (δ, $CHCl_3$) ppm: 1.63 (d, J=18.9 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 270.2104 [M^+, $C_{15}{}^{13}C_1H_{23}{}^2H_3O_3$ requires 270.2104] (3), 269 (0.2), 252 (5), 236 (2), 220 (9), 193 (5), 167 (100).

4.1.1.3. Deprotection of [15-$^{13}C^2H_3$]-artemisinic acid methyl ester (14a) yielding [15-$^{13}C^2H_3$]-artemisinic acid (2a). To a solution of 15-[$^{13}C^2H_3$]-artemisinic acid methyl ester (**14a**) (126 mg, 0.50 mmol) in MeOH (10 ml) was added an aqueous solution of KOH (180 mg, 3.2 mmol; 10 ml) and the reaction mixture was heated to reflux overnight. After cooling and acidification to pH 4 (HCl, 10%), the mixture was extracted by $CHCl_3$ (3×20 ml). The combined organic layers were washed with brine (3×5 ml), dried ($MgSO_4$), and solvent was removed under reduced pressure to yield [15-$^{13}C^2H_3$]-artemisinic acid (**2a**) without the need for further purification.

*4.1.1.3.1. [15-$^{13}C^2H_3$]-Artemisinic acid (**2a**).* Oil (102 mg; 86%) (see also Refs. 9 and 10 and Section 4.5.1 for physical properties). IR ν (cm^{-1}): 3400–2600 (m), 2924 (m), 2870 (m), 1692 (s), 1622 (m), 1435 (m), 1279 (s), 1221 (m). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.45 (1H, s, H-13), 5.55 (1H, s, H-13), 4.99 (1H, d, J=6.5 Hz, $^3J_{CH}$, H-5), 2.67 (1H, ddd, J=12.4, 4.1, 4.1 Hz, H-7), 2.61 (1H, br s, H-6), 0.89 (3H, d, J=5.8 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm: 172.7 C (C-12), 142.7 C (C-11), 134.9 C (d, J=43.0 Hz, $^1J_{CC}$, C-4), 126.6 CH_2 (C-13), 120.2 CH (C-5), 42.1 CH (C-7), 41.4 CH (C-1), 38.0 CH (d, J=4.0 Hz, $^3J_{CC}$, C-6), 35.3 CH_2 (C-9), 27.6 CH (C-10), 26.4 CH_2 (d, J=3.4 Hz, $^2J_{CC}$, C-3), 26.0 CH_2 (C-8), 25.7 CH_2 (d, J=3.0 Hz, $^3J_{CC}$ C-2), 22.9 ((1:3:6:7:6:3:1) sept., J=19.1 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.7 CH_3 (C-14)—see also Table 1; ^{2}H NMR (δ, $CHCl_3$) ppm: 1.58 (d, J=19.1 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 238.1850 [M^+, $C_{14}{}^{13}C_1H_{19}{}^2H_3O_2$ requires 238.1842] (94), 237 (6), 236 (2), 235 (0.5), 220 (18), 210 (16), 209 (9), 193 (25), 192 (22), 166 (14), 153 (15), 140 (35), 125 (100), 123 (86).

4.1.2. Introduction of the $\Delta^{11,13}$ double bond in *epi*-deoxyarteannuin B (9) by oxidative selenation of dihydro-*epi*-deoxyarteannuin B (15). To dry THF (3 ml) cooled to −78 °C under an atmosphere of N_2 were added distilled diisopropylamine (0.16 m, 1.14 mmol) and *n*-butyllithium (0.7 ml, 1.6 M in hexane, 1.12 mmol). The mixture was stirred for 1 h, then dihydro-*epi*-deoxyarteannuin B (**15**) (0.2 g, 0.85 mmol) in THF (1 ml) was added and stirring continued for a further 30 min. Distilled hexamethylphosphoramide (0.3 ml) was then added and the mixture stirred for another 30 min before raising the temperature to −10 °C and stirring for a further 2 h. The reaction mixture

was cooled to −78 °C and a solution of phenylselenyl bromide (0.189 g, 0.80 mmol) in THF (2 ml) was added and stirring continued for 15 min. The temperature was then increased to −10 °C and stirring was continued for another 1 h before acetic acid (1 ml; 25%) and H_2O_2 (0.5 ml; 30% in H_2O) were added and the mixture allowed to warm to room temperature, and then stirred for 30 min. The reaction mixture was extracted by Et_2O (3×50 ml) and the organic layers were combined and washed by saturated $NaHCO_3$ (3×10 ml), followed by brine (3×10 ml), dried ($MgSO_4$), and solvent was removed in vacuo to yield a crude product (0.29 g), consisting of compounds **20** and **9**, which were separable by HPLC (silica column, 5% EtOAc/*n*-hexane).

4.1.2.1. *epi*-Deoxyarteannuin B (9). Oil (42 mg, 0.18 mmol, 21%; t_R 19.5 min—see Ref. 6 for NMR properties, and Refs. 17 and 18 for other physical properties; see also Section 4.5.5). HREIMS *m*/*z* (rel int.): 232.1463 [M^+, $C_{15}H_{20}O_2$ requires 232.1464] (100), 217 (30), 204 (11), 187 (53), 173 (35).

4.1.2.2. 7,11-Dehydro-dihydro-*epi*-deoxyarteannuin B (20). Oil (78 mg, 0.34 mmol, 39%; t_R 26.1 min). IR ν (cm^{-1}): 3017 (m), 2945 (m), 2862 (m), 1732 (s), 1682 (m), 1454 (m), 1261 (m). ^1H NMR (δ, $CDCl_3$) ppm: 5.40 (1H, d, *J*=1.3 Hz, H-5), 2.63 (1H, dd, *J*=12.7, 9.1 Hz, H-8), 2.30–2.10 (3H, m, H-3 and H-8), 1.82 (3H, s, H-13), 1.69 (3H, s, H-15), 1.41 (1H, ddd, *J*=12.6, 10.1, 10.1 Hz, H-9), 0.84 (3H, d, *J*=6.7 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm—see Table 1; HREIMS *m*/*z* (rel int.): 232.1458 [M^+, $C_{15}H_{20}O_2$ requires 232.1464] (75), 217 (28), 204 (26), 177 (23), 153 (100), 136 (82).

4.1.3. Introduction of the $\Delta^{11,13}$ double bond in artemisitene (4) by oxidative selenation of artemisinin (1). To dry THF (15 ml) cooled to −10 °C was added diisopropylamine (1 ml, 7.1 mmol), followed by *n*-butyllithium (1.4 ml, 1.6 M in hexane, 2.24 mmol). The reaction mixture was stirred for 10 min and then cooled to −78 °C, before the addition of a solution of artemisinin (**1**) (0.56 g, 1.97 mmol) in THF (5 ml). Stirring was continued for another 30 min, then a solution of phenylselenyl bromide (0.345 g, 1.46 mmol) in THF (5 ml) was transferred to the reaction mixture, which was stirred at −78 °C for a further 2 h. Solutions of acetic acid (2 ml; 25% in H_2O) and H_2O_2 (1 ml; 50% in H_2O) were added and stirring was continued at room temperature for a further 30 min, before the mixture was extracted by Et_2O (3×50 ml), washed by saturated $NaHCO_3$ (3×5 ml) followed by brine (3×10 ml), dried ($MgSO_4$), and solvent removed in vacuo to yield a crude product (0.64 g).

4.1.3.1. Artemisitene (4). Yield 5% (0.03 g, 0.11 mmol)—see Refs. 13–15 for physical properties.

4.1.3.2. 11-e*pi*-Artemisinin (21). Oil (0.41 g, 1.45 mmol, 73%). IR ν (cm^{-1}): 2966 (m), 2931 (m), 2878 (m), 1732 (s), 1450 (w), 1379 (m), 1107 (s). ^1H NMR (δ, $CDCl_3$) ppm: 5.93 (1H, s, H-5), 2.39 (1H, ddd, *J*=13.4, 13.4, 3.9 Hz, H-3), 2.28 (1H, q, *J*=7.4 Hz, H-11), 2.08 (1H, m, H-3), 1.94 (1H, m, H-2), 1.80 (1H, dddd, *J*=13.4, 3.6, 3.6, 3.6 Hz, H-8), 1.71 (1H, dddd, *J*=13.1, 3.6, 3.5, 3.5 Hz, H-9), 1.47 (3H, d, *J*=7.4 Hz, H-13), 1.46 (3H, s, H-15), 1.12 (1H, dddd, *J*=13.3, 13.3, 13.3, 3.6 Hz, H-9), 1.00 (3H, d, *J*=5.9 Hz, H-14)—see also Table 2; ^{13}C NMR (δ, $CDCl_3$) ppm—see Table 1; EIMS *m*/*z* (rel int.): 250 [M^+-O_2] (26), 236 (15), 232 (17), 209 (32), 192 (100), 180 (34), 152 (72).

4.2. *A. annua* plants

A. annua plants were grown outdoors from seed (seeds were supplied by Prof. Guan-Yi Liang from Guizhou province, Guiyang, and taxonomically verified specimens are held by him; a voucher specimen of plant material grown from the seeds (no. HKU2285) has also been deposited in The University of Hong Kong herbarium collection). Plants were used for this feeding experiment after approximately three months growth, when mature and beginning to come into flower.

4.2.1. Preparation of a feeding solution of [15-$^{13}C^2H_3$]-artemisinic acid (2a) in TRIS buffer. The maximum solubility of compound **2a** in TRIS buffer (50 mM, pH 8.1) was found to be around 1.3 mg/ml after ultrasonification for 2–3 h. ^1H NMR analysis indicated no change in the composition of **2a** as a result of ultrasonification and previous studies[33] had determined that this concentration of TRIS buffer caused no discernible physical injury to intact *A. annua* plants. Therefore, feeding solutions were prepared, which contained around 1 mg of **2a**/1 ml buffer.

4.3. 'Stem-feeding' of 2a to cut *A. annua* plants

Forty-two *A. annua* plants were cut just above the root and weighed individually (fresh plant weights were in the range 10–30 g). Individual plants were then fed with the labeled precursor at a rate of approximately 1 mg of **2a**/10 g fresh plant material, by immersing the cut stem in a vial containing 2 ml of the feeding solution (using a solution prepared from 90 mg of **2a** in 90 ml of TRIS buffer (50 mM, pH 8.1)). 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, 'stem-cut' plants were normally able to take up all the feeding solution over a period of 4–6 h. A small amount of TRIS buffer was then added to the feeding vial in order to rinse any remaining labeled precursor from the walls of the vial, and the plants were left for a short while longer to take up this additional small volume.

Half of the plants were then kept alive outdoors for a period of 1 day to one week by continually replenishing their feeding vials with deionized water (hydroponic conditions). For the other half, the feeding vials were not replenished with water, once dry, and the plants in this group were therefore allowed to die by desiccation. The ambient temperature was 21–27 °C over the course of the experiment and the skies were mostly sunny.

4.3.1. Extraction procedures. Extracts of the 42 individual plants were made at 1, 2, 3, 4, 5, 6, and 7 days after the termination of feeding. The total plant weight at extraction for the live group was found to be between 60 and 85% of the initial weight that had been determined before feeding; this slight loss in weight may indicate some dehydration as a result of the forcing conditions, which were employed in

order to stimulate the transpiration stream and to promote uptake of the feeding solution. For the 'dead' group, the plant weight had dropped to 30% of the original weight after 3 days as a result of desiccation.

Each plant was extracted separately by grinding to a fine powder under liquid 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 to prevent any spontaneous autoxidation by oxygen), which was then placed on a shaker for 8 h. The extraction vessels were filtered free of plant particulates, and the solvent was dried ($MgSO_4$) and removed in vacuo to yield a crude plant extract. This extraction procedure was repeated again overnight (without shaking) and the two CH_2Cl_2 extracts were combined. It was generally found that the efficiency of extraction with CH_2Cl_2 was between 1 and 2% (w/w). The crude CH_2Cl_2 extract from each plant was analyzed immediately after extraction (in order to minimize the possibility of autoxidation) by both ^2H and ^1H NMR spectroscopies.

All the plant residues were then re-extracted by MeOH (100 ml) using similar procedures to those described above (the efficiency of extraction in MeOH was between 2 and 5% (w/w)). Some recovery of ^2H label was also noted in these MeOH extracts, although this was consistently less than that of the corresponding CH_2Cl_2 extracts. However, ^2H NMR analysis of the MeOH extracts indicated only very broad poorly defined resonances (unlike the CH_2Cl_2 extracts, which are discussed in Section 4.4—see also Fig. 3, for an example), and these samples were not analyzed further.

4.4. ^2H NMR analysis of the crude CH_2Cl_2 extracts from the aerial parts of *A. annua*, which had been fed with 2a

The initial analysis of each individual *A. annua* plant that had been fed with **2a** 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 both reference and a standard for quantitative calibration (see Section 4.1). The extent of recovery of ^2H label in each CH_2Cl_2 extract was calculated by comparing the ratio of the total integral in the aliphatic region of each ^2H NMR spectrum to that of the internal calibration standard, C_6D_6 at δ_D 7.43 ppm (Section 4.1).

4.4.1. ^{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 2a. The crude CH_2Cl_2 extracts from all three members of a triplicate group of plants, which had been harvested and extracted on the same day were combined into a single sample (ca. 400 mg), which was re-dissolved in ca. 0.6 ml $CDCl_3$ and analyzed by ^{13}C–^2H COSY spectroscopy with the AV 600 instrument (see Section 4.1).

4.5. Separation of labeled metabolites from the CH_2Cl_2 extracts of the aerial parts of *A. annua*, which had been fed with 2a

All the CH_2Cl_2 extracts of the 21 plants of *A. annua*, which had been fed with **2a** and allowed to die by desiccation (the 'dead' group) were combined together (total weight: 2.56 g) and subjected to a 'preliminary' HPLC separation using the diol column (18% EtOAc/*n*-hexane) from which 20 fractions were collected over a 50 min period.[††] A total of 16 injections were made—each injection containing approximately 150 mg of the crude plant material—and the HPLC column was 'washed' with 50% EtOAc/*n*-hexane/1% AcOH between injections, in order to obtain a 'polar residue' fraction (1.2 g), created from the combined washings. Those chromatographic fractions from the 'preliminary' separation, which exhibited a doublet signal in their ^2H NMR spectra were subjected to a second round of HPLC on the silica column, for which the polarity of the mobile phase was adjusted to match the polarity of the fraction under study.

The 'polar residue' from the 'preliminary' HPLC was also re-subjected to HPLC separation on a silica column (50% EtOAc/*n*-hexane/1% AcOH): 13 injections were made (ca. 100 mg per injection), resulting in the collection of 20 fractions over a 50-min period. Those fractions, which exhibited a doublet signal in their ^2H NMR spectra, corresponding to more polar metabolites of **2a**, were then further purified in a second round of HPLC (silica column again), with the polarity of the mobile phase being adjusted according to the polarity of the fraction under study.

The eight labeled metabolites, which were isolated from these extensive preparative HPLC separations are listed in Sections 4.5.1–4.5.8.

4.5.1. [15-$^{13}C^2H_3$]-Artemisinic acid (2a)/artemisinic acid (2). Oil (3.1 mg, t_R 25–27 min in the preliminary separation (diol HPLC column); t_R 15.0 min with 17% EtOAc/*n*-hexane (silica HPLC column)). (See also Section 4.1.1.3.1 for physical properties). ^1H NMR (δ, $CDCl_3$) ppm: 6.43 (1H, s, H-13), 5.55 (1H, s, H-13), 4.99 (1H, br s), 2.69 (1H, d, J=12.1 Hz, H-7), 2.60 (1H, br s, H-6), 1.59 (ca. 85% of [3H], for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 0.90 (3H, d, J=5.8 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 170.9 (C-12), 142.4 (C-11), 135.0 (C-4), 126.4 (C-13), 120.2 (C-5), 42.2 (C-7), 41.4 (C-1), 37.9 (C-6), 35.2 (C-9), 27.6 (C-10), 26.4 (C-3), 25.9 (C-8), 25.6 (C-2), 23.7 (s, isotopically-normal 15-CH_3), 22.8 ((1:3:6:7:6:3:1) sept., J=19.1 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 19.7 (C-14); ^2H NMR (δ, $CHCl_3$) ppm: 1.58 (d, J=19.1 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 238.1840 [M^+, $C_{14}{}^{13}C_1H_{19}{}^2H_3O_2$ requires 238.1842] (10), 237 (1), 236 (0.2), 234.1612 [M^+, $C_{15}H_{22}O_2$ requires 234.1620] (56), 206 (14), 189 (26), 121 (100), 119 (77).

4.5.2. [15-$^{13}C^2H_3$]-Arteannuin B (3a)/arteannuin B (3). Oil (1.5 mg, t_R 22–25 min in the preliminary (diol HPLC column); t_R 58.3 min in 17% EtOAc/*n*-hexane (silica HPLC column)). ^1H NMR (δ, $CDCl_3$) ppm: 6.15 (1H, d, J=3.2 Hz, H-13a), 5.43 (1H, d, J=3.2 Hz, H-13b), 2.74 (1H, dddd, J=12.3, 3.2, 3.2, 3.2 Hz, H-7), 2.68 (1H, s, H-5), 2.06 (1H, dddd, J=13.3, 3.2, 3.2, 3.2 Hz, H-8), 1.90 (2H, m, H-3 and H-8), 1.76 (1H, ddd, J=13.3, 13.3, 6.2 Hz, H-2), 1.69 (1H, ddd, J=13.9, 13.3, 4.6 Hz, H-3), 1.52 (1H, m, H-2), 1.49 (2H, m, H-1 and H-10), 1.41 (1H,

[††] The 21 CH_2Cl_2 plant extracts from the 'live' plant experiment were also combined together (total weight 2.40 g) and subjected to a separate HPLC separation, which gave similar results to those reported herein for the 'dead' group.

dddd, J=13.3, 12.6, 12.3, 3.4 Hz, H-8), 1.33 (ca. 80% of [3H], for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 1.25 (1H, m, H-9), 0.99 (3H, d, J=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 169.9 (C-12), 138.6 (C-11), 117.5 (C-13), 81.0 (C-6), 58.6 (C-5), 58.3 (C-4), 52.7 (C-7), 43.7 (C-1), 33.9 (C-9), 30.6 (C-10), 24.3 (C-3), 22.7 (s, isotopically-normal 15-CH_3), 21.9 ((1:3:6:7:6:3:1) sept., J=19.2 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$), 21.7 (C-8), 18.5 (C-14), 16.2 (C-2); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.33 (d, J=19.3 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 252.1629 [M^+, $C_{14}{}^{13}C_1H_{17}{}^2H_3O_3$ requires 252.1634] (2), 248.1409 [M^+, $C_{15}H_{20}O_3$ requires 248.1412] (17), 230 (49), 206 (96), 190 (100), 177 (88), 163 (70), 162 (65).

4.5.3. [15-$^{13}C^2H_2$]-Annulide (7a)/annulide (7) (inseparable from isoannulide (8)). Oil (0.2 mg, t_R 14–15 min in the preliminary separation (diol HPLC column); t_R 22.3 min in 10% EtOAc/*n*-hexane (silica HPLC column)). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.51 (1H, s, H-13a), 5.60 (1H, s, H-13b), 5.20 (ca. 80% of 1H, s, for a mixture of isotopically-normal 15-H_2 and isotopically-labeled 15-D_2), 5.03 (1H, d, J=11.4 Hz, H-5), 4.88 (1H, ca. 80% of 1H, s, for a mixture of isotopically-normal 15-H_2 and isotopically-labeled 15-D_2), 2.66 (1H, ddd, J=11.9, 5.5, 5.5 Hz, H-7), 2.26 (1H, ddd, J=13.9, 3.4, 3.4 Hz, H-3α), 2.17 (1H, dd, J=13.9, 3.0 Hz, H-3β), 2.04 (1H, m, H-2β), 2.01 (1H, m, H-6), 1.85 (1H, m, H-9β), 1.70 (1H, m, H-10), 1.69 (2H, m, H-8α and H-8β), 1.56 (1H, m, H-1), 1.45 (1H, m, H-2α), 1.17 (1H, dddd, J=12.5, 12.5, 12.5, 3.7 Hz, H-9α), 0.92 (3H, d, J=6.6 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 165.1 (C-12) not seen due to small amount of sample, 145.3 (C-4), 139.2 (C-11), 129.1 (C-13), 106.5 (s, isotopically-normal 15-CH_2), 106.0 ((1:2:3:2:1) quin., J=24 Hz, $^1J_{CD}$, 15-$^{13}C^2H_2$), 75.5 (C-5), 43.3 (C-6), 42.7 (C-1), 40.4 (C-7), 34.8 (C-9), 30.5 (C-8), 29.3 (C-3), 28.7 (C-2), 27.9 (C-10), 20.0 (C-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 5.24 (1H, d, J=24.7 Hz, $^1J_{CD}$, 15a-D_2), 4.92 (1H, d, J=23.5 Hz, $^1J_{CD}$, 15b-D_2); HREIMS m/z (rel int.): 235.1615 [$C_{14}{}^{13}C_1H_{18}{}^2H_2O_2$ requires 235.1622] (8), 232.1463 [$C_{15}H_{20}O_2$ requires 232.1463] (100), 217 (22), 204 (11), 175 (8).

4.5.4. [15-$^{13}C^2H_3$]-Isoannulide (8a)/isoannulide (8) (inseparable from annulide (7)). Oil (0.1 mg, t_R 14–15 min in the preliminary separation (diol HPLC column); t_R 22.3 min in 10% EtOAc/*n*-hexane (silica HPLC column)). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.53 (1H, s, H-13a), 5.62 (1H, s, H-13b), 5.47 (1H, br s, H-3), 4.84 (1H, d, J=10.5 Hz, H-5), 2.68 (1H, ddd, J=11.6, 5.1, 5.1 Hz, H-7), 2.22 (2H, m, J= H-2α and H-2β), 2.19 (1H, m, H-6), 1.82 (70% of 3H, s, for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 1.79 (1H, m, H-8α), 1.78 (1H, m, H-9β), 1.69 (1H, m, H-8β), 1.52 (1H, m, H-1), 1.31 (1H, m, H-10), 1.13 (1H, dddd, J=12.3, 12.3, 12.3, 3.9 Hz, H-9α), 0.89 (3H, d, J=6.1 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 166.7 (C-12) not seen due to small amount of sample, 140.0 (C-11) not seen due to small amount of sample, 130.5 (C-4), 129.2 (C-13), 124.1 (C-3), 74.7 (C-5), 40.9 (C-1), 39.4 (C-6), 38.6 (C-7), 34.8 (C-9), 30.5 (C-8), 29.0 (C-10), 26.9 (C-2), 20.2 (C-14), 18.5 (s, isotopically-normal 15-CH_3), 17.6 ((1:3:6:7:6:3:1) sept., J=19.0 Hz, $^1J_{CD}$, 15-$^{13}C^2H_3$); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.81 (d, J=19.0 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 236.1673 [$C_{14}{}^{13}C_1H_{17}{}^2H_3O_2$ requires 236.1685] (3).

4.5.5. [15-$^{13}C^2H_3$]-*epi*-Deoxyarteannuin B (9a)/*epi*-deoxyarteanuin B (9). Oil (0.8 mg, t_R 13–14 min in the preliminary separation (diol HPLC column); t_R 22.2 min in 9% EtOAc/*n*-hexane (silica HPLC column)). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.17 (1H, s, H-13a), 5.56 (1H, s, H-13b), 5.28 (1H, s, H-5), 2.71 (1H, dd, J=6.6, 6.6 Hz, H-7), 2.10 (1H, dd, J=18.1, 6.2 Hz, H-3α), 2.03 (1H, m, H-3β), 1.89 (1H, m, H-8α), 1.86 (1H, m, H-2α), 1.66 (70% of 3H, s, for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 1.67 (2H, m, H-2β and H-9β), 1.56 (1H, m, H-10), 1.38 (1H, m, H-8β), 1.25 (1H, ddd, J=10.5, 10.5, 2.7 Hz, H-1), 1.12 (1H, m, H-9α), 0.97 (3H, d, J=6.9 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 169.9 (C-12), 142.9 (C-11), 141.3 (C-4), 123.8 (C-5), 120.6 (C-13), 83.4 (C-6), 44.9 (C-7), 44.3 (C-1), 31.1 (C-3), 30.0 (C-9), 28.5 (C-8), 28.4 (C-10), 23.2 (s, isotopically-normal 15-CH_3), 22.3 ((1:3:6:7:6:3:1) sept., J=19.2 Hz, 15-$^{13}C^2H_3$), 21.7 (C-2), 20.0 (C-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.66 (d, J=19.2 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 236.1677 [$C_{14}{}^{13}C_1H_{17}{}^2H_3O_2$ requires 236.1686] (25), 235 (3), 234 (0.5), 232.1462 [$C_{15}H_{20}O_2$ requires 232.1464] (100), 217 (28), 191 (13), 188 (35), 187 (47), 173 (25).

4.5.6. [15-$^{13}C^2H_3$]-Deoxyarteannuin B (10a)/deoxyarteannuin B (10). Oil (0.1 mg, t_R 13–14 min in the preliminary separation (diol HPLC column); t_R 23.7 min in 9% EtOAc/*n*-hexane (silica HPLC column)). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.09 (1H, d, J=3.2 Hz, H-13a), 5.36 (1H, d, J=3.2 Hz, H-13b), 5.13 (1H, s, H-5), 2.66 (1H, dddd, J=11.7, 2.7, 2.7, 1.5 Hz, H-7), 2.04 (1H, dddd, J=18.1, 10.2, 6.9, 2.7 Hz, H-2α), 1.94 (2H, m, H-3α and H-3β), 1.93 (1H, m, H-8α), 1.84 (1H, m, H-2β), 1.80 (1H, m, H-9β), 1.67 (70% of 3H, s, for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 1.66 (1H, m, H-1), 1.56 (1H, m, H-10), 1.35 (1H, dddd, J=12.6, 12.6, 12.6, 3.7 Hz, H-8β), 0.99 (3H, d, J=6.6 Hz, H-14); ^{13}C NMR (δ $CDCl_3$) (ppm): 170.8 (C-12) not seen due to small amount of sample), 142.5 (C-4) not seen due to small amount of sample, 139.9 (C-11) not seen due to small amount of sample), 118.8 (C-5), 116.8 (C-13), 89.4 (C-6) not seen due to small amount of sample, 52.5 (C-7), 44.8 (C-1), 34.3 (C-9), 30.7 (C-10), 26.2 (C-3), 23.6 (s, isotopically-normal 15-CH_3), 22.7 ((1:3:6:7:6:3:1) sept., J=19.1 Hz, 15-$^{13}C^2H_3$), 22.1 (C-8), 19.9 (C-2), 19.8 (C-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.67 (d, J=19.1 Hz, $^1J_{CD}$,15-D_3); HREIMS m/z (rel int.): 236.1684 [$C_{14}{}^{13}C_1H_{17}{}^2H_3O_2$ requires 236.1686] (21), 235 (2), 232.1460 [$C_{15}H_{20}O_2$ requires 232.1464] (80), 217 (19), 187 (100), 173 (29), 153 (81), 136 (53).

4.5.7. [15-$^{13}C^2H_3$]-*seco*-Cadinane (13a)/*seco*-cadinane (13). Oil (0.4 mg, t_R 18–21 min in the first HPLC of the 'polar residue' (silica HPLC column); t_R 37.2 min in 35% EtOAc/*n*-hexane/0.5% AcOH (silica HPLC column)). ^{1}H NMR (δ, $CDCl_3$) ppm: 9.36 (1H, br s, H-5), 6.39 (1H, s, H-13a), 5.70 (1H, s, H-13b), 2.83 (1H, br, H-7), 2.45 (1H, m, H-3a), 2.34 (1H, m, H-3b), 2.33 (1H, m, H-6), 2.12 (80% of 3H, s, for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 1.88 (1H, br s, H-9β), 1.86 (1H, m, H-2a), 1.80 (1H, d, J=10.5 Hz, H-8), 1.51 (1H, m, H-2b), 1.34 (1H, m, H-9α), 1.31 (2H, m, H-1 and H-10), 1.21 (H-8β, dddd, J=13.2, 11.6, 11.6, 2.6 Hz, H-8β), 0.96 (3H, d, J=6.2 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) (ppm): 208.5 (C-5), 204.2 (C-4), 170.3 (C-12), 141.4

(C-11), 128.3 (C-13), 57.9 (C-6), 41.7 (C-1), 39.8 (C-7), 38.2 (C-3), 34.9 (C-2), 33.4 (C-10), 32.0 (C-9), 29.9 (s, isotopically-normal 15-CH_3), 29.0 ((1:3:6:7:6:3:1) sept., J= 19.4 Hz, 15-$^{13}C^2H_3$), 23.5 (C-8), 19.7 (C-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 2.12 (d, J=19.4 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 252.1635 [$C_{14}{}^{13}C_1H_{17}{}^2H_3O_3$ requires 252.1634] (12), 251 (1), 250 (0.5), 248.1414 [$C_{15}H_{20}O_3$ requires 248.1412] (72), 230 (85), 220 (40), 205 (100), 202 (35), 192 (77), 178 (87).

4.5.8. [15-$^{13}C^2H_3$]-Artemisinic acid methyl ester (14a)/ artemisinic acid methyl ester (14). Oil (0.1 mg, t_R 9–10 min in the preliminary separation (diol HPLC column); t_R 14.3 min in 2% EtOAc/*n*-hexane (silica HPLC column)). (See also Ref. 5 and Section 4.1.1.2.1). ^{1}H NMR (δ, $CDCl_3$) ppm: 6.30 (1H, s, H-13a), 5.46 (1H, s, H-13b), 5.01 (1H, br s, H-5), 3.77 (3H, s, 15-OMe), 2.74 (1H, d, J=12.1 Hz, H-7), 2.57 (1H, s, H-6), 1.62 (80% of 3H, s, for a mixture of isotopically-normal 15-H_3 and isotopically-labeled 15-D_3), 0.92 (3H, d, J=5.9 Hz, H-14); ^{13}C NMR (δ, $CDCl_3$) ppm: 167.8 (C-12) not seen due to small amount of sample, 143.3 (C-11) not seen due to small amount of sample, 134.7 (C-4) not seen due to small amount of sample, 124.1 (C-13), 120.3 (C-5), 51.7 (15-OMe), 42.4 (C-7), 41.4 (C-1), 38.0 (C-6), 35.3 (C-9), 27.6 (C-10), 26.4 (C-3), 25.9 (C-8), 25.6 (C-2), 23.7 (s, isotopically-normal C-15), 22.8 ((1:3:6:7:6:3:1) sept., $^1J_{CD}$=19.1 Hz, 15-$^{13}C^2H_3$), 19.7 (C-14); ^{2}H NMR (δ, $CHCl_3$) ppm: 1.62 (d, J=19.1 Hz, $^1J_{CD}$, 15-D_3); HREIMS m/z (rel int.): 252.1992 [$C_{15}{}^{13}C_1H_{21}{}^2H_3O_2$ requires 252.1998] (7), 251 (25), 248.1773 [$C_{16}H_{24}O_2$ requires 248.1776] (48), 230 (7), 220 (10), 216 (25), 189 (38), 153 (100).

Acknowledgements

We thank the Generic Drug Research Programme of the Chemistry Department of The University of Hong Kong for providing a postdoctoral fellowship to Dr. L.-K.S. The seeds of *A. annua* were kindly supplied by Prof. Liang of the Guiyang laboratory of natural products, Guizhou province, China. This project was funded by a grant from the Committee on Conference and Research Grants (CRCG).

References and notes

1. Haynes, R. K.; Vonwiller, S. C. *Acc. Chem. Res.* **1997**, *30*, 73–79.
2. Tu, Y.-Y.; Ni, M.-Y.; Zhong, Y.-R.; Li, L.-N.; Cui, S.-L.; Zhang, M.-Q.; Wang, X.-Z.; Ji, Z.; Liang, X.-T. *Planta Med.* **1982**, *44*, 143–145.
3. Brown, G. D. *Educ. Chem.* **2006**, *43*, 97–99.
4. Sy, L.-K.; Brown, G. D.; Haynes, R. *Tetrahedron* **1998**, *54*, 4345–4356.
5. Sy, L.-K.; Brown, G. D. *Phytochemistry* **1998**, *48*, 1207–1211.
6. Sy, L.-K.; Brown, G. D. *Phytochemistry* **2001**, *58*, 1159–1166.
7. Brown, G. D.; Liang, G.-Y.; Sy, L.-K. *Phytochemistry* **2003**, *64*, 303–323.
8. Sy, L.-K.; Cheung, K.-K.; Zhu, N.-Y.; Brown, G. D. *Tetrahedron* **2001**, *57*, 8481–8493.
9. Misra, L. N.; Ahmad, A.; Thakur, R. S.; Lotter, H.; Wagner, H. *J. Nat. Prod.* **1993**, *56*, 215–219.
10. Kim, S.-U.; Han, J.; Lim, Y.-H. *Planta Med.* **1996**, *62*, 480–481.
11. Zheng, G.-Q. *Planta Med.* **1994**, *60*, 54–57.
12. Jeremic, D.; Jokic, A.; Behbud, A.; Stefanovic, M. *Tetrahedron Lett.* **1973**, 3039–3042.
13. Acton, N.; Klayman, D. L. *Planta Med.* **1985**, *51*, 441–442.
14. El-Feraly, F. S.; Ayalp, A.; Al-Yahya, M. A.; McPhail, D. R.; McPhail, A. T. *J. Nat. Prod.* **1990**, *53*, 66–71.
15. Sy, L.-K.; Brown, G. D. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2421–2429.
16. Zhu, D.-Y.; Deng, D.-A.; Zhang, S.-G.; Xu, R.-S. *Acta Chim. Sin.* **1984**, *42*, 937–939.
17. Brown, G. D. *Phytochemistry* **1993**, *32*, 391–393.
18. Roth, R. J.; Acton, N. *Planta Med.* **1987**, *53*, 576.
19. El-Feraly, F. S.; Al-Meshal, I. A.; Khalifa, S. I. *J. Nat. Prod.* **1989**, *52*, 196–198.
20. Wu, Z.-H.; Wang, Y.-Y. *Acta Chim. Sin.* **1984**, *42*, 596–598.
21. Brown, G. D. *Phytochemistry* **1994**, *36*, 637–641.
22. Wang, Y.; Xia, Z.-Q.; Zhou, F.-Y.; Wu, Y.-L.; Huang, J.-J.; Wang, Z.-Z. *Acta Chim. Sin.* **1988**, *46*, 1152–1153.
23. Sangwan, R. S.; Agrawal, K.; Luthra, R.; Thakur, R. S.; Singh-Sangwan, N. *Phytochemistry* **1993**, *34*, 1301–1302.
24. Huang, J.-J.; Zhou, F.-Y.; Wu, L.-F.; Zhen, G.-H. *Acta Chim. Sin.* **1990**, 275–277.
25. Wang, Y.; Xia, Z.-Q.; Zhou, F.-Y.; Wu, Y.-L.; Huang, J.-J.; Wang, Z.-Z. *Chin. J. Chem.* **1993**, *11*, 457–463.
26. Wang, Y.; Shen, Z.-W.; Xia, Z.-Q.; Zhou, F.-Y. *Chin. J. Chem.* **1993**, *11*, 476–478.
27. Brown, G. D.; Sy, L.-K. *Tetrahedron* **2007**, *63*, 9536–9547 (as a companion paper with the current manuscript).
28. Nair, M. S. R.; Basile, D. V. *J. Nat. Prod.* **1993**, *56*, 1559–1566.
29. Nair, M. S. R.; Basile, D. V. *Indian J. Chem.* **1992**, *31B*, 880–882.
30. Akhila, A.; Thakur, R. S.; Popli, S. P. *Phytochemistry* **1987**, *26*, 1927–1930.
31. Brown, G. D.; Sy, L.-K. *Tetrahedron* **2004**, *60*, 1125–1138.
32. Sy, L.-K.; Zhu, N.-Y.; Brown, G. D. *Tetrahedron* **2001**, *57*, 8495–8510.
33. Brown, G. D.; Sy, L.-K. *Tetrahedron* **2004**, *60*, 1139–1159.
34. Zhou, W.-S.; Huang, D.-Z.; Ping, X.-F.; Zhang, L.; Zhu, J.; Xu, X.-X. *Acta Chim. Sin.* **1989**, *47*, 710–715.
35. Zhou, W.-S. *Pure Appl. Chem.* **1986**, *58*, 817–824.
36. Sy, L.-K.; Brown, G. D. *Tetrahedron* **2002**, *58*, 897–908.
37. Sy, L.-K.; Brown, G. D. *Tetrahedron* **2002**, *58*, 909–923.
38. Zhang, L.; Zhou, W.-S. *Acta Chim. Sin.* **1989**, *47*, 1117–1119.
39. Brown, G. D. *Phytochem. Rev.* **2003**, *2*, 45–59.
40. Brown, G. D. *^{13}C–^2H Correlation Spectroscopy: Development and Applications to Chemical and Biological Problems*, 15th International Meeting on NMR spectroscopy, University of Durham, July 8–12, 2001.
41. Wallaart, T. E.; van Uden, W.; Lubberink, H. G. M.; Woerdenbag, H. J.; Pras, N.; Quax, W. J. *J. Nat. Prod.* **1999**, *62*, 430–433.
42. Wallaart, T. E.; Pras, N.; Quax, W. J. *J. Nat. Prod.* **1999**, *62*, 1160–1162.
43. Bertea, C. M.; Freije, J. R.; van der Woude, H.; Verstappen, F. W. A.; Perk, L.; Marquez, V.; de Kraker, J.-W.; Posthumus, M. A.; Jansen, B. J. M.; de Groot, Aa.; Franssen, M. C. R.; Bouwmeester, H. J. *Planta Med.* **2005**, *71*, 40–47.
44. Teoh, K. H.; Polichuk, D. R.; Reed, D. W.; Nowak, G.; Covello, P. S. *FEBS Lett.* **2006**, *580*, 1411–1416.
45. El-Feraly, F. S.; Al-Mesha, I. A.; Al-Yahya, M. A.; Hifnawy, M. S. *Phytochemistry* **1986**, *25*, 2777–2778.
46. Hayes, R. K.; Vonwiller, S. C. *J. Chem. Soc., Chem. Commun.* **1990**, 451–453.

47. Acton, N.; Roth, R. J. *J. Org. Chem.* **1992**, *57*, 3610.
48. Chen, D.-H.; Ye, H.-C.; Li, G.-F. *Plant Sci.* **2000**, *155*, 179–185.
49. Bouwmeester, H. J.; Wallart, T. E.; Janssen, M. H. A.; van Loo, B.; Jansen, B. J. M.; Posthumus, M. A.; Schmidt, C. O.; de Kraker, J.-W.; Konig, W. A.; Franssen, M. C. R. *Phytochemistry* **1999**, *52*, 843–854.
50. Chang, Y.-J.; Seung, S.-H.; Park, S.-H.; Kim, S.-U. *Arch. Biochem. Biophys.* **2000**, *383*, 178–184.
51. Martin, V. J. J.; Pitera, D. J.; Withers, S. T.; Newman, J. D.; Keasling, J. D. *Nat. Biotechnol.* **2003**, *21*, 796–802.
52. Ro, D.-K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C. Y.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D. *Nature* **2006**, *440*, 940–943.
53. Wallaart, T. E.; Pras, N.; Beekman, A. C.; Quax, W. J. *Planta Med.* **2000**, *66*, 57–62.