Studies on the Mechanism of Polyketide-derived Biosynthesis of Deoxyradicinin and Related Metabolites of *Alternaria helianthi*

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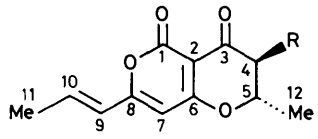
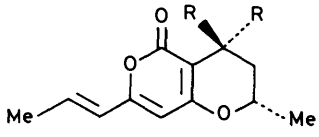
The polyketide biosynthetic origin of deoxyradicinin, deoxyradicinol and 3-epideoxyradicinol has been demonstrated by incorporation studies with ^{13}C-labelled precursors and ^{13}C n.m.r. spectroscopy. The ^{13}C n.m.r., ^{2}H n.m.r., and mass spectra of deoxyradicinin isolated from fungal cultures grown in the presence of [$^{2}H_3$]sodium acetate indicated an uncommon condensation of two polyketide chains rather than a single hexaketide unit undergoing cyclization and ring-cleavage.

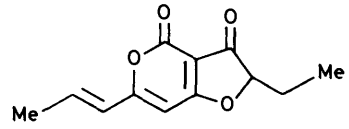
The filamentous fungus *Alternaria helianthi* (Hansf.) Tubaki and Nishihara [1] is a widely-distributed, destructive pathogen of *Helianthus annuus* L. (sunflower),[2,3] which is itself an agronomic crop plant of global economic importance.[4] Four phytotoxic compounds which are structurally related to radicinin (**1**),[5,6] as well as radicinin itself, have been isolated from liquid cultures of this fungus [7–9] while the major component, deoxyradicinin (**2**), has also been isolated from the infected plant.[10] Previous investigations by Seto and Urano [11] and Tanabe *et al.*[12] on the biosynthesis of radicinin from *A. radicina* (syn. *Stemphylium radicinum*) have shown that radicinin is derived from a polyketide origin. In their investigations Seto and Urano [11] used this knowledge to assist in the assignment of signals in the ^{13}C n.m.r. spectrum of radicinin. An earlier study by Robeson and Strobel [8] on deoxyradicinin similarly demonstrated the polyketide origin of this compound. Thus when the fungus *A. helianthi* was grown on a medium containing [1-^{13}C]- or [2-^{13}C]-acetate the intensities of the ^{13}C n.m.r. signals ascribed to C-1,3,5,6,8,10 and C-2,4,9,11,12, respectively, of compound (**2**) were greatly enhanced. Polyketide fungal metabolites which can be formed by the folding of a single polyketide chain are normally assumed to arise in this manner. However, given the established polyketide origin of radicinin (**1**) and its derivatives (**2**), (**3**), (**4**), and (**5**) we can conceive three distinct biosynthetic schemes for the assembly of the carbon skeleton of these metabolites. For example, biosynthesis could be achieved *via* fusion of a C_4 and a C_8, or two C_6 fragments. A third alternative is the assembly of the molecule from a single hexaketide chain involving cyclization to a pre-hexylphenyl derivative followed by C-2/C-3 or C-5/C-6 fission (Scheme 1). Data reported in previous studies using doubly labelled [^{13}C]acetate as precursor [11,12] did not distinguish between any of the three above alternative biosynthetic mechanisms. In the present investigation, incorporation of [^{13}C]-and [$^{2}H_3$]-acetate was studied in an attempt to define the precise pathway of biosynthesis utilizing the extraordinarily high degree of incorporation of acetate displayed by the fungus.



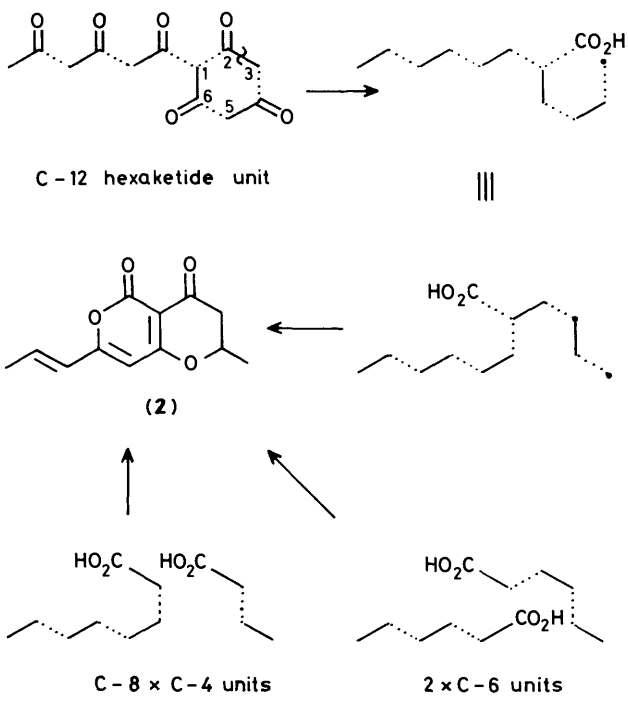
(**1**) R = OH Radicinin
(**2**) R = H Deoxyradicinin



(**3**) R^1 = OH, R^2 = H 3-Epideoxyradicinol
(**4**) R = H, R = OH Deoxyradicinol



(**5**) Radianthin



Scheme 1. Alternative biogenetic pathways of deoxyradicinin

In our first approach to this problem, we incubated the fungus with an equimolar mixture of [1-^{13}C]- and [2-^{13}C]-acetate. As expected, this resulted in the general enhancement of signals in the ^{13}C n.m.r. spectrum of the resulting metabolites (**2**)—(**5**). In those instances where linkages between carbon atoms derived from different acetate units (i.e. inter-acetate linkages) occurred, the carbon atoms involved were identified, and the intensities of the satellite peaks accounted for approxi-

Table. ^{13}C N.m.r. chemical shifts, δ (p.p.m.), and ^{13}C–^{13}C spin–spin coupling constants (Hz) observed in the ^{1}H noise decoupled spectra of deoxyradicinin (**2**), 3-epideoxyradicinol (**3**), and deoxyradicinol (**4**) from *A. helianthi* grown in the presence of a mixture of [1-^{13}C]- and [2-^{13}C]-enriched sodium acetate

	Deoxyradicinin (**2**)		3-Epideoxyradicinol (**3**)		Deoxyradicinol (**4**)	
Carbon[a]	δ	$J^{1}_{^{13}C-^{13}C}$	δ	$J^{1}_{^{13}C-^{13}C}$	δ	$J^{1}_{^{13}C-^{13}C}$
C-1	157.2		164.4		164.2	
C-2	100.1	58; 62[a]	102.0	48.9; 67.9	102.4	47.1; 68.0
C-3	186.4	57.8	58.8	49.0	61.2	46.7
C-4	43.7	33.8	36.3	35.4	36.5	35.5
C-5	76.5	34.3	70.6	35.2	73.7	35.5
C-6	175.9	62.5	166.1	68.1	165.6	67.5
C-7	98.1	71.4	99.2	73.8	99.2	74.2
C-8	163.4	71.4	158.4	73.8	158.3	74.0
C-9	122.6	71.0	122.7	71.9	122.6	71.9
C-10	139.9	71.2	135.0	71.9	135.1	71.6
C-11	18.6		18.4		18.4	
C-12	20.3		20.3		20.7	

[a] Approximate values; satellites were blurred due to long range coupling to ^{13}C-4.

mately 25% that of the original signal. This result indicates that incorporation of endogenous acetate may be insignificant compared with that of added acetate precursor, a hypothesis supported by the extremely high levels of incorporation which we have consistently observed in these studies. These intensities of the satellite peaks are consistent with the expected values based on the probability associated with the juxtaposition of two labelled carbons (Figure 1). The use of mixed [1-^{13}C]- and

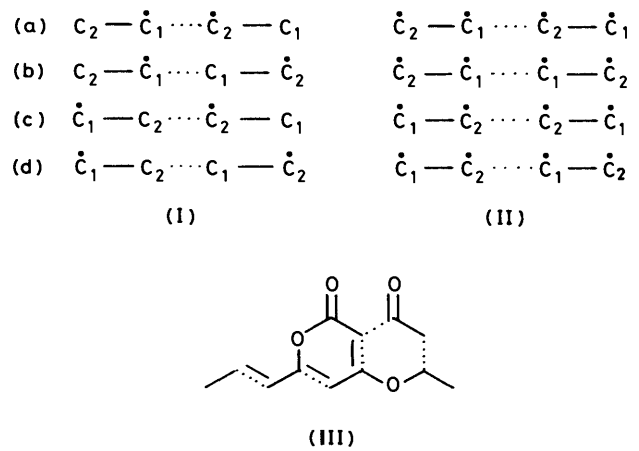


Figure 1. Representative inter-acetate linkages resulting from use of (I) mixed singly labelled and (II) doubly labelled [^{13}C]-acetate. Fed intact acetate units are represented as (C–C) and biosynthetically linked inter-acetate units as (C · · · C)

[2-^{13}C]-labelled acetate, as opposed to doubly labelled acetate, eliminated vicinal coupling associated with every doubly labelled acetate unit incorporated into the molecule, resulting in a simpler ^{13}C n.m.r. spectrum from which the inter-acetate linkages were readily identified.

The ^{13}C–^{13}C spin–spin coupling constants of the inter-acetate linkages revealed that acetate had been incorporated into the following pairs of carbon atoms in deoxyradicinin (**2**), 3-epideoxyradicinol (**3**), and deoxyradicinol (**4**): C-1/C-2, C-3/C-4, C-5/C-12, C-6/C-7, C-8/C-9, and C-10/C-11; *cf.* Table. Radicinin (**1**) and radianthin (**5**)[9] were isolated in insufficient quantities to allow assignments of the acetate subunits although the linkages are fully expected and assumed to be as in compounds (**2**)—(**4**). Yields of labelled metabolites were comparable to those described in reference 9.

The signal for C-2 of deoxyradicinin biosynthesized from mixed singly labelled acetate, and of the other compounds (**3**) and (**4**), appears as a doublet of doublets (Table and Figure 2). Since labelling with mixed acetate highlights the inter-acetate linkages, these data demonstrate that C-2 is attached to two other acetate units and therefore represents the point of juncture of three inter-acetate units. In contrast, the use of a double labelled acetate precursor would not have demonstrated this feature. The use of mixed singly labelled [1-^{13}C]- and [2-^{13}C]-acetate, instead of doubly labelled [1,2-^{13}C]acetate units, to highlight interacetate linkages is substantiated by the results shown in Figure 2. Using an equimolar mixture of singly labelled acetate greatly enhances, by the same proportion, the n.m.r. signals of all carbons in this representative diketide well above the background signals of the natural ^{13}C product. However, of the four possible representations for the diketide (**1a—d**) (Figure 1) only (**1a**) would be expected to show observable vicinal ^{13}C–^{13}C coupling. The use of doubly labelled [1,2-^{13}C]acetate, as is customary in such studies, would also proportionally enhance the n.m.r. signal of all carbons. However in this case, if we consider the vicinal couplings, only ^{13}C–^{13}C coupling of intact acetate units would be significant. Therefore, the final product of deoxyradicinin biosynthesis from labelled acetate may be represented by structure III (Figure 1). Thus, our data show that C-2 of deoxyradicinin originates either from C-1 of the C_{12} hexaketide unit (Scheme 1) or from the linking methylene carbon in a mixed polyketide pathway. Furthermore, since signals for both C-12 and C-11 of deoxyradicinin appear as singlets, neither could have arisen as a result of C-2/C-3 cleavage of the C_{12} hexaketide unit. Although a C-5/C-6 cleavage is possible, cleavage exclusively at this position seems highly unlikely in view of the symmetry of the hexaketide unit. On the basis of the above, the mixed polyketide biosynthetic pathway is favoured.

Further support for our hypothesis that deoxyradicinin is formed from two distinct polyketide units comes from a study in which the ^{13}C n.m.r. spectrum of deoxyradicinin (**2**), isolated from *A. helianthi* grown in the presence of [$^{2}H_3$]-labelled acetate, showed the presence of deuterium in the 11- and 12-Me by exhibiting upfield β-isotope shifts.[13,14] Thus, the influence of three deuterium atoms attached to the 12-Me group on the chemical shift of the C-5 carbon atom was clearly demonstrated by the three characteristic resonances, or β-isotope shifts (0.035 p.p.m. each) upfield of the major signal at δ 76.5 for C-5. Similarly, the β-isotope shifts (0.030 p.p.m. each) accompanying the C-10 signal at δ 139.9 indicated that C-11 was also deuteri-

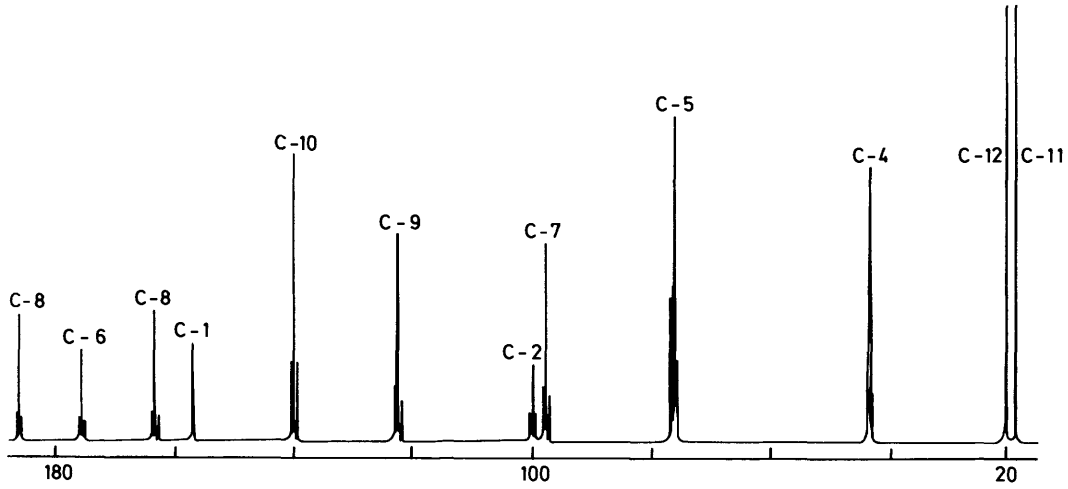


Figure 2. 75 MHz proton noise-decoupled ^{13}C n.m.r. spectrum in $CDCl_3$ of deoxyradicinin derived from equimolar mixture of [1-^{13}C]- and [2-^{13}C]-acetate

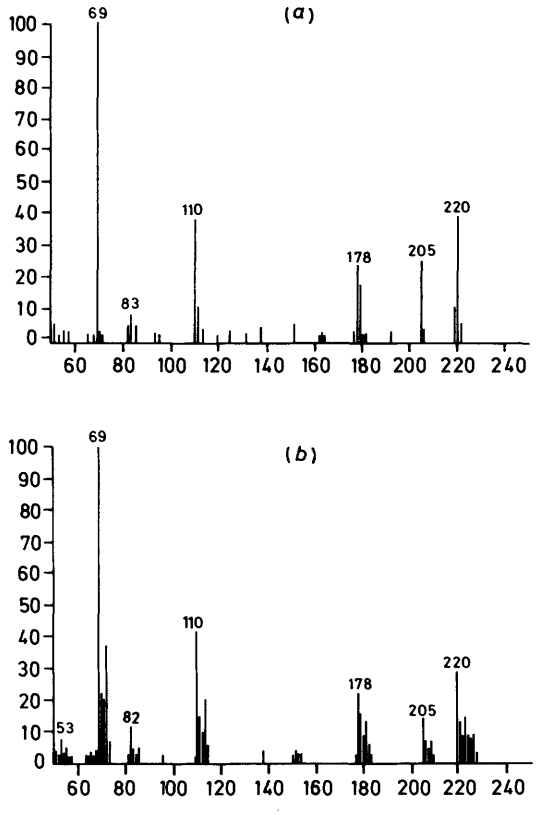
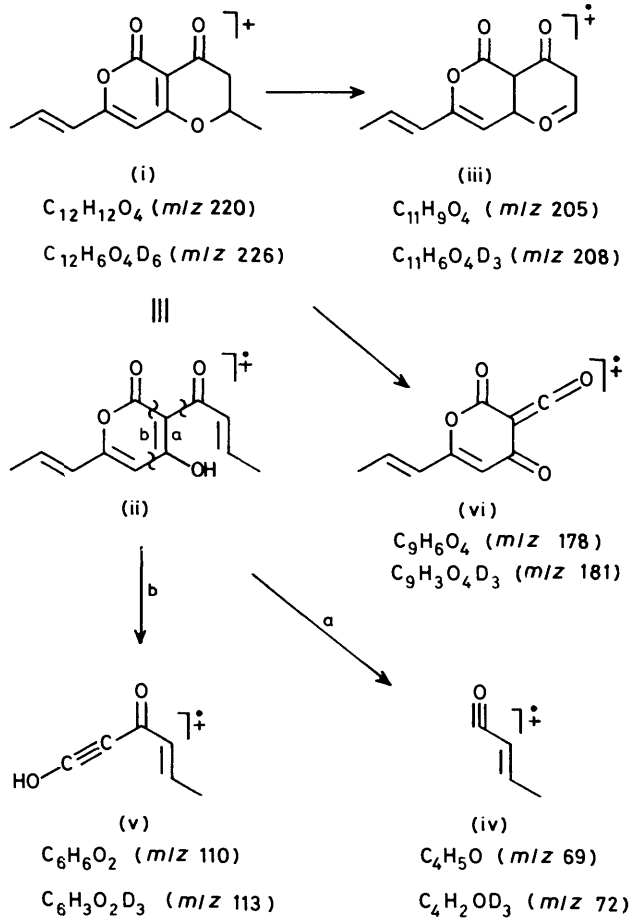


Figure 3. Mass spectra of unlabelled (*a*) and deuterium labelled (*b*) deoxyradicinin

ated, although in this case the three resonances were incompletely resolved. The absence of β-isotope shifts for signals of C-8, C-6, and C-3 failed to detect the presence of deuterons at C-9, C-7, and C-4. This indicated a total exchange or 'wash out'[15] of deuterium atoms on the active methylenes in the proposed intermediate polyketide units.

Further evidence for the selective retention of deuterium by the molecule employed mass spectrometry, and took advantage of the extraordinarily high degree of incorporation of acetate precursor into this metabolite (**2**). Thus the selective retention of deuterium at the 11-and 12-Me groups was readily apparent from a comparison of the m.s. and h.r.m.s. of unlabelled deoxyradicinin with those recorded for the deuterium-labelled compound (Figure 3). The molecular ion *m/z* 220 ($C_{12}H_{12}O_4$) of the unlabelled compound was shifted to *m/z* 226 ($C_{12}H_6D_6O_4$) in the case of ^{2}H-labelled deoxyradicinin, thus signalling the presence of up to six deuterium atoms in the molecule (Figure 3). The $[M - Me]^+$ fragments (Figure 3 and Scheme 2) in the



Scheme 2. M.s. fragmentation of deoxyradicinin

spectra of labelled and unlabelled deoxyradicinin (208 and 205, respectively) (Scheme 2) firmly indicate the presence of three deuterium atoms in the 12-Me group. Furthermore, the but-2-enoyl acylium ion [Scheme 2(iv)], *m/z* 69 (base peak), which contains the 12-Me group is shifted to *m/z* 72 ($C_4H_2D_3O$), thereby providing additional support that in the labelled deoxyradicinin there are no deuterium atoms at C-4. The loss of a single methyl group bearing three deuterium atoms clearly shows that C-12 of deoxyradicinin originated from an acetate 'starter' unit.[16] When considered in relation to the n.m.r. data, the retention of the remaining three deuterium atoms in fragments (iii) and (vi) further signifies that the 11-Me group contains the three remaining deuterium atoms. These data are consistent with the previously observed rapid exchange of deuterium atoms from active methylene groups during the biosynthesis of polyketides from [$^2H_3$]acetate precursor.[15] In the final product these centres contain only protons, while the terminal Me groups of the polyacetate chains selectively retain deuterium.

The above conclusion, that both the 11- and 12-Me groups originate from acetate 'starter' units, and the postulate that deoxyradicinin evolved *via* two distinct polyketide moieties was vindicated by the results obtained from observing the ^2H n.m.r. spectrum of the sample of deuteriated deoxyradicinin used for mass spectral studies. The broad band proton decoupled spectrum of this deuterio-enriched sample indicated two signals of approximately equal integral. No other signals were observed in this spectrum. Subsequently, when the two signals were referenced against internal deuteriochloroform at 7.25 p.p.m., their chemical shifts were found to be 1.96 and 1.53 p.p.m. These values are virtually identical with those obtained for 11- and 12-Me (1.95 and 1.54 p.p.m. respectively) in the ^1H n.m.r. spectrum of undeuteriated deoxyradicinin.[7] It is well known that there is no significant difference between the chemical shifts of protons and their deuterium analogues.[17–19] Thus the presence of these signals at 1.96 and 1.53 together with the remarkable absence of any other signals in the ^2H n.m.r. spectrum of deoxyradicinin obtained from culturing *A. helianthi* in the presence of [$^2H_3$]acetate unequivocally demonstrates that the only significant retention of deuterons in the sample of deuteriated deoxyradicinin occurred at C-11 and C-12. This result therefore confirms our findings, inferred from ^{13}C n.m.r. and mass spectrometry, that C-11 and C-12 originate from acetate 'starter' units, and in addition, that deuterons attached to active methylenes of the polyketide units were totally 'washed out.' Most significantly our results demonstrate for the first time that the radicinins are derived from the linking of two distinct polyketide fragments.

Grove,[20] who used ^{14}C-labelled precursors in feeding experiments with *A. radicina*, also favoured condensation of two, possibly identical C_6 fragments, linking at positions 2 and 6 of radicinin. This would be accompanied by cyclization. No likely C_8 precursor molecules were detected by Grove[20] in the culture filtrate of *A. radicina*, the fungus used in his investigation. Our results, while confirming that biosynthesis occurs *via* the fusion of two polyketide chains, did not however distinguish between the two alternative fragment condensations referred to above, since both would be linked *via* C-2 of deoxyradicinin.

In an effort to further resolve the mechanism of biosynthesis, the suitability of the C_6 polyketide fragment as a precursor was examined. Thus, synthetic [1-^{13}C]-3,5-dioxohexanoic acid was fed to 1-week liquid cultures of the fungus as a potential late precursor of deoxyradicinin. However, no significant enhancement in any of the resonances in the spectrum of the resulting sample of the metabolite was observed as compared with the natural abundance spectrum. Despite repeated attempts, incorporation of the synthetic C_6 compound was never observed. Possible explanations for lack of incorporation of this potential precursor include decarboxylation, incorporation into the lipid components of the mycelium, or simply a failure of the compound to act as substrate for an enzyme involved in deoxyradicinin formation.

These results, particularly those derived from the utilization of deuterium labelling, demonstrate that deoxyradicinin is biosynthesized *via* an uncommon condensation of two polyketide fragments, as opposed to, and thereby excluding from further consideration, a single hexaketide precursor. Although the fate of the [1-^{13}C]-3, 5-dioxohexanoic acid precursor is at present unknown, this compound represents a potential probe for the precise elucidation of the interesting mechanism of polyketide juncture involved in the biosynthesis of deoxyradicinin and related metabolites.

Experimental

Labelled Acetate Precursors.—[1-^{13}C]- and [2-^{13}C]-sodium acetate were purchased from KOR. [^2H]sodium acetate was obtained from Merck, Sharpe, and Dohme. All precursors were 99% enriched.

Feeding of Labelled Acetate and Isolation of ^{13}C- *and* ^2H-*Labelled Phytotoxins.*—*Alternaria helianthi* was grown in 300 ml batches of modified Czapek–Dox broth in 1 litre Erlenmeyer flasks under conditions previously described.[6] At day 7, ^{13}C-labelled sodium acetate (total 1 g; 0.5 g [2-^{13}C]- and 0.5 g [1-^{13}C]-NaOAc), or $^2H_3$-labelled NaOAc (1 g) were added per 900 ml of culture. Incubation was continued for a further 7 days and at day 14 culture filtrates were extracted with $CHCl_3$. Labelled toxins were purified by preparative t.l.c. (silica gel) first in Et_2O to afford compounds (**2**), (**3**), and (**4**), R_F 0.13, 0.39, and 0.48, respectively and secondly in hexane–Me_2CO (2:1), R_F 0.17, 0.27, and 0.38, respectively.

^1H- *and* ^{13}C-*N.m.r. Spectroscopy.*—A Varian XL-300 spectrometer was used to record the ^1H- and ^{13}C-n.m.r. spectra at 300 and 75.4 MHz, respectively. All spectra were obtained in $CDCl_3$ solutions using TMS as internal reference. ^{13}C N.m.r. data of the ^{13}C-labelled metabolites (**2**), (**3**), and (**4**) are given in the Table. The ^{13}C n.m.r. spectrum of ^2H-labelled deoxyradicinin (**2**) revealed β-isotope shifts of −0.105 (3 × 0.035) and −0.089 (3 × 0.030) p.p.m., respectively, for the signals at δ 76.5 (C-5) and δ 139.9 (C-10).

^2H *N.m.r. Spectroscopy.*—The ^2H n.m.r. spectrum was recorded on a Varian XL300 instrument at 40.66 MHz, in $CHCl_3$ ± trace amount of $CDCl_3$ as internal standard (δ 7.25). The pulse width was 5.0 μs and the FIDS were collected in 576 data points with a spectral width of 590 Hz.

Mass Spectrometry.—Low and high resolution mass spectra were recorded on a VG 7070E instrument in the e.i. mode at 70 eV with direct probe insertion. The significant h.r.m.s. fragments of unlabelled and ^2H-labelled deoxyradicinin, respectively, are as follows: *m/z* 220.074 (28%, $C_{12}H_{12}O_4$), 226.111 (9, $C_{12}H_6O_4D_6$); 205.050 (14, $C_{11}H_9O_4$), 208.069 (5, $C_{11}H_6O_4D_3$); 178.027 (22, $C_9H_6O_4$), 181.045 (13, $C_9H_3O_4D_3$); 110.037 (42, $C_6H_6O_2$), 113.056 (20, $C_6H_3O_2D_3$); 69.034 (100, C_4H_5O), and 72.053 (37, $C_4H_2D_3O$).

Assignment of ^{13}C *Resonances.*—Deoxyradicinin (**2**). The assignments are based on selective ^1H decoupling experiments, and the multiplicities observed in the off-resonance ^1H decoupled spectrum.

3-Epideoxyradicinol (**3**). The assignments are derived from the J^1 ^{13}C–^{13}C coupling constants observed for ^{13}C-labelled (**3**); *cf.* Table.

Deoxyradicinol (**4**). The assignments are based on multiplicities in the off-resonance ^1H decoupled spectrum for synthetic (**4**),[8] and the J^1 ^{13}C–^{13}C spin–spin coupling constants observed for ^{13}C-labelled (**4**); *cf.* Table.

Synthesis of ^{13}C-*Labelled* 3,5-*Dioxohexanoic Acid.*—To a flame dried, argon flushed two-necked round bottom flask fitted with septa, was added dry THF (50 ml) directly from an LAH still. The flask was cooled to 0 °C in an ice bath and flushed with argon. Di-isopropylamine (5.2 ml; 0.037 mol) was added dropwise *via* syringe followed by butyl-lithium (14.8 ml of a 2.5M solution in hexane; 0.037 mol). Within 5 min, acetylacetone (1.2 ml; 0.012 mol) was added slowly, dropwise, and the mixture was stirred at 0 °C for 15 min before addition by double tipped needle to a sealed flask containing ^{13}C-labelled CO_2 (g) (250 ml; 0.01 mol) which was cooled in a separate ice bath. The resulting yellow slurry was allowed to stand for 15 min with occasional agitation, and then condensed *in vacuo* to give a yellow solid (6.7 g). The yellow salt was dissolved in ice-H_2O (50 ml) and acidified (6M HCl) first to pH 7.0 to remove unchanged starting material, then to pH 2.5. The organic material was extracted into EtOAc (6 × 25 ml), dried ($MgSO_4$), and concentrated with minimal heat by rotary evaporation to give a reddish liquid (1.5 g) which after purification by column chromatography (silica gel 62, hexane–EtOAc) yielded the pure acid (0.8 g, 55%) as a yellow liquid which solidified in the freezer. $\delta_H(CDCl_3)$ 2.09 (3 H, s, Me), 3.40 (2 H, d, $J^{2}_{^{13}C\ H}$ 7.7 Hz, CH_2), and 5.6 (1 H, s, =CH); $\delta_C(CDCl_3)$ 24.01 (C-6), 44.32 (d, $J_{^{13}C-^{13}C}$ 56.4 Hz, C-2), 100.57 (C-4), 171.45 (s, together with satellites, d, $J_{^{13}C_1-^{13}C_2}$ 56.2 Hz, centred at 171.45, C-1), 187.68 (C-3), and 189.96 (C-5); *m/z* (rel int) 146 (14) $[M + H]^+$, 100 (100) $[M + H - COOH]^+$.

Feeding of ^{13}C-*Labelled* 3,5-*Dioxohexanoic Acid and Isolation of Deoxyradicinin.*—[1-^{13}C]-3,5-Dioxohexanoic acid (600 mg) was added aseptically to a 7 day liquid shake culture of *A. helianthi* (600 ml). After incubation as given above for an additional 7 days, deoxyradicinin (7.5 mg) was isolated from the culture filtrate as described and subjected to ^{13}C n.m.r. analysis.

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