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INTER- AND INTRAMOLECULAR ISOTOPIC CORRELATIONS IN SOME CYANOGENIC GLYCOSIDES AND GLUCOSINOLATES AND THEIR PRACTICAL IMPORTANCE*

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Key Word Index—*Sinapis alba*; *Brassica nigra*; Cruciferae; ^{13}C-pattern; glucosinolates; cyanogenic glycosides; kinetic isotope effects; intermolecular ^{13}C-correlation; sinalbin; sinigrin; biosynthesis.

Abstract—Sinalbin is a complex organic salt from white mustard. The δ ^{13}C-values of the aromatic parts of the anion glucosinalbin (*p*-hydroxyphenylacetic acid) and of the cation sinapin (sinapic acid) are identical (−32.2‰) and both aromatic compounds are depleted by 6.4‰ with respect to the glucose moiety (−25.8‰) bound in glucosinalbin. The δ ^{13}C-value of the choline part of the molecule can be correlated to its metabolic origin from glucose. However, there is an unexpected dramatic ^{13}C-enrichment in the first C-atom of glucosinalbin, originally the C-2 of tyrosine. This enrichment of approximately 11‰ relative to the mean value of the aglycone is found in the same position of four other glucosinolates and cyanogenic glycosides derived from phenylalanine or tyrosine. An isotope effect on the phenylalanine–ammonia-lyase reaction is discussed as the most probable cause for this finding. In contrast, sinigrin, the glucosinolate from black mustard, shows a relative ^{13}C-depletion of 6.5‰ in the corresponding first C-atom of the aglycone allyl mustard oil. The proposed reason for this depletion is an isotope effect on an aldol reaction during the biosynthetic introduction of this C-atom into the aglycone skeleton. Synthetic allyl mustard oil is depleted by 14‰ in the same position relative to the δ ^{13}C-value of the whole molecule, probably owing to an isotope effect on the first step of its chemical synthesis, the binding of allyl amine to CS_2. This difference can be used to detect adulterations of mustard. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Intermolecular ^{13}C-correlations between natural compounds reflect metabolic correlations and indicate their origin from a common precursor. In general, primary products of metabolism are 'heavier', while secondary products are relatively depleted in ^{13}C by characteristic values, e.g. lipids and/or aromatic compounds 3–6 δ-units relative to carbohydrates from the same origin [1]§. In addition, typical intramolecular distributions (patterns) are observed in any natural compound [2]. The main reason for these findings are kinetic isotope effects on enzymatic key reactions. However, these can, in a closed system such as a plant, lead to isotopic discriminations only when they occur at metabolic branching points. In this case we find depletions of a given compound or position and, for compensation, corresonding enrichments of the metabolic counterpart or complementary position [3].

Amino acids are the starting materials for many secondary products such as alkaloids or other heterocyclic compounds. These may partially preserve the pattern of their precursors but acquire, in addition, shifts in their isotopic pattern, owing to isotope effects in the secondary metabolism. An interesting group of amino acid derivatives are cyanogenic glycosides and glucosinolates. In addition to the general interest in isotope fractionations in the course of their biosynthesis, some of these compounds or their hydrolysis products have importance as food and spice ingredients and are therefore targets for adulteration. We therefore studied the intermolecular and intramolecular ^{13}C-distributions of representatives of these groups and possible reasons for them.

*Preliminary results of this paper are referred to in a review (see [2]).

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§The δ^{13}C-value is defined by: $\delta^{13}C\ [‰] = \left(\frac{[^{13}C]/[^{12}C]_{sample}}{[^{13}C]/[^{12}C]_{PDB}} - 1\right) \times 1000$ PDB is a carbonate standard with a carbon isotope ratio of $[^{13}C]/[^{12}C] = 0.0112372$.

RESULTS

*Intermolecular correlations of compounds from white mustard (*Sinapis alba*)*

The most important ingredient of white mustard seed (*Sinapis alba*) is sinalbin, a salt of the glucosinolate glucosinalbin and of sinapin, an ester of choline with sinapic acid (Fig. 1). The core of both compounds is an aromatic molecule, synthesized via the shikimic acid pathway. Glucosinalbin contains glucose as part of its molecule; this moiety will serve in this study as an 'intermolecular primary standard', because it is a representative of the primary product pool in the source investigated. The choline moiety of sinapin is synthesized from ethanolamine, readily derived from serine, which is closely related to carbohydrates in biosynthesis. Finally, *N*- and *O*-methyl groups present in sinapin originate from the pool of C-1 units, which are also related to serine metabolism, and therefore indirectly to glucose [4]. Sinalbin should therefore be an ideal test compound for comparison of the metabolic and isotopic correlations of different pathways within a given plant. Hydrolysis of the products and the δ ^{13}C-values of the fragments confirmed this correlation.

As expected, secondary compounds, especially the aromatic moieties, were markedly depleted relative to glucose, and the δ ^{13}C-values of both aromatic cores, probably originating from the same precursor tyrosine, were practically identical. For a discussion of the isotope pattern of the choline part, one has to make the following assumptions: The ethanolamine skeleton is correlated to C-atoms (1 + 2 and 6 + 5) of glucose via serine. From the earlier reported pattern of this precursor (C-1 and C-6 are relatively depleted, C-3 and C-4 enriched, [5]) one can assume that choline (atoms 2 + 1 and 5 + 6) is depleted relative to glucose (δ ^{13}C = −25.8‰) by 1.8 to 2.0‰, and thus would attain a δ ^{13}C-value of −27.6‰. On this basis, by difference, a value of −32.8‰ would result for the methyl groups. This negative value generally agrees with the observation that methyl groups (originating from *S*-adenosylmethionine, SAM) are relatively depleted [4, 6–8]. If we attribute the same value to the methoxyl groups of sinapic acid, a value of −32.4‰

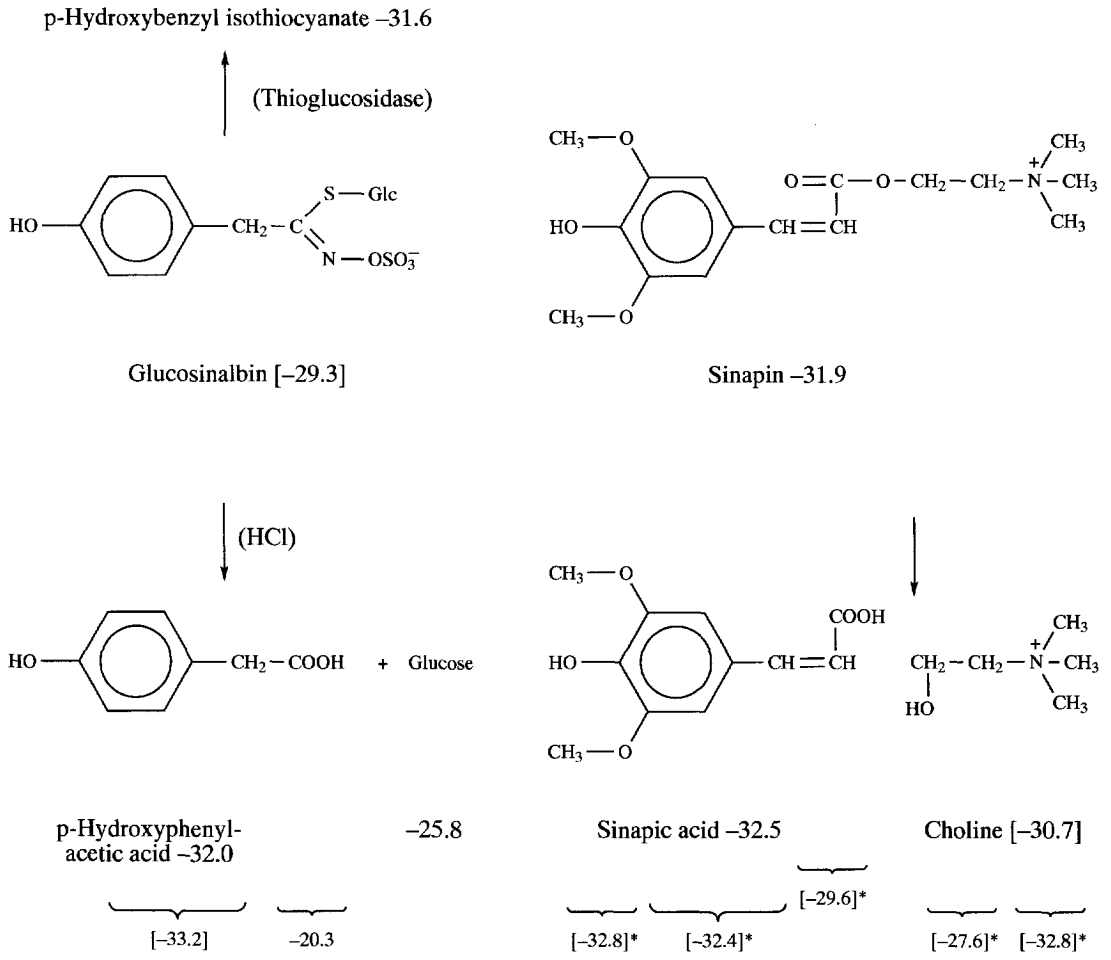


Fig. 1. δ ^{13}C values $[‰]_{PDB}$ of sinalbin (glucosinalbin/sinapin) from white mustard seed, and of its hydrolysis and degradation products. Values in [ ] calculated from differences, in [ ]* estimated from other results (see text).

will result for the phenylpropane moiety, which is very close to the measured value of *p*-hydroxyphenylacetic acid from glucosinalbin (−32.0‰). Therefore the intermolecular (and partial intramolecular) ^{13}C-pattern of these compounds is in good agreement with their metabolic origins.

Partial isotope pattern of cyanogenic glycosides and glucosinolates originating from aromatic amino acids

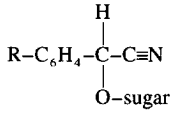
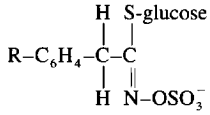
Phenylalanine and tyrosine are the precursors not only of glucosinalbin, but also of other glucosinolates and of various cyanogenic glycosides. The enzymatic hydrolysis of these compounds liberates the C-atom 1 (originally the C-2 atom of the side-chain of the amino acid) as HCN. Hydrolysis to formic acid and subsequent oxidation to CO_2 provides the opportunity to compare the δ ^{13}C-value of this C-atom with that of the carboxyl atom of pyruvic acid from which it is formed in the shikimate pathway. We therefore isolated aromatic glucosinolates and cyanogenic glycosides from various plants, determined the total δ ^{13}C-values of the sugar and the aglycone moieties, and of the C-atom in question (Table 1).

The data show that position C-2, corresponding to the α-C-atom of the aromatic amino acid precursor, is dramatically enriched in ^{13}C, regardless of whether the compound was obtained from a C-3 or a C-4 plant, or from phenylalanine or tyrosine, or whether the glycoside was a cyanogenic compound or a glucosinolate. These findings may be discussed on the basis of the following alternative explanations (Fig. 2).

(1) The pattern of the precursor pyruvate (phosphoenolpyruvate) could not be that expected from glucose (small relative depletion in position 2, [5]); instead, a secondary ^{13}C- enrichment in position 2 could occur, owing to partial conversion of the pyruvate pool to acetyl-CoA by the pyruvate dehydrogenase reaction, which implies an isotope effect on position 1 and 2 of pyruvate [9]. However, one can assume that the pyruvate pool for this reaction is not identical with that of the precursors of the shikimic acid pathway. This is supported by the fact that phenylalanine isolated from a protein hydrolysate of bitter almonds, from which amygdalin (Table 1) was obtained, showed qualitatively the pattern in the side-chain expected from glucose and 'normal' pyruvate (Table 1).

(2) Phenylalanine (and tyrosine) themselves constitute a pool, from which different pathways with different turnover rates originate, the major one starting with

Table 1. Partial ^{13}C-pattern in cyanogenic glycosides and glucosinolates derived from phenylalanine or tyrosine (δ ^{13}C in $[‰]_{PDB}$)

Structure, name and origin of compound	δ ^{13}C [‰] in molecule part				
	Glycoside	Sugar	Aglycon	C_6C_1	C-2
					
Amygdalin (R = H); (bitter almonds). sugar = gentiobiose	−24.8	−22.9	−28.1 [−28.4	−29.7 −29.2	−16.8 −26.9*]†
Sambunigrin (R = H); (elderberry leaves). sugar = glucose	−29.1*	−26.1	−31.4	−33.2	−19.1
Dhurrin (R = OH); (durra germs). sugar = glucose	−16.2*	−15.2	−16.9	−17.7	−11.5
					
Glucotropeolin (R = H); (garden cress). sugar = glucose	−28.6*	−25.4	−31.1	−32.8	−19.0
Glucosinalbin (R = OH); (white mustard seed); sugar = glucose	−29.3*	−25.8	−32.0	−33.2	−20.3

* δ ^{13}C values calculated from those of the other atoms and the total value.

† Values for the complete side chain of phenylalanine from the same origin (mean δ $^{13}C = -28.4‰$): C-1, −24.9‰; C-2, −26.9‰*; C-3, −28.8‰; C_6-rest (aromate), −29.6‰*. For comparison δ ^{13}C-value shifts ($\Delta\delta$ ^{13}C-values) from the mean value of pyruvate, precursor of the side chain: C-1, +4.2‰; C-2, −0.9‰; C-3, −3.2‰ [5].

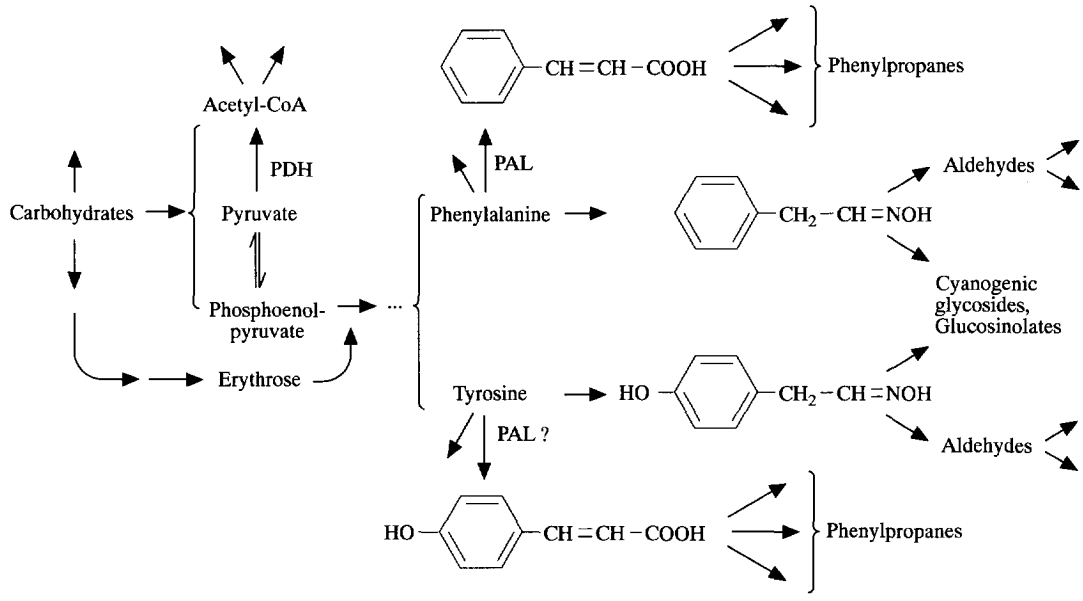


Fig. 2. Pathways and branching points in the biosynthesis of the carbon skeleton of cyanogen glycosides and glucosinolates from phenylalanine and tyrosine. Enzyme-catalysed reactions with discussed isotope discriminations are indicated. PDH = pyruvate dehydrogenase (decarboxylating); PAL = phenylalanine ammonia lyase.

the phenylalanine–ammonia-lyase (PAL) reaction. If this reaction involves a carbon isotope effect on C-atom 2, one would have to expect a relative ^{13}C-depletion in the position C-2 for all phenylpropane compounds (e.g. lignin), and, for compensation, an enrichment in other products originating directly from the aromatic amino acids such as the cyanogenic glycosides and glucosinolates discussed here. A metabolic balance demands that when these are minor products of the total pool (as is probably the case here), their enrichment in position 2 would be remarkable, while the depletion in the main products would not be so dramatic.

Because of experimental difficulties, the pattern of sinapic acid has not been thoroughly investigated. However, the following calculation could support the hypothesis. If we assume that both of the aromatic components of mustard seed glucosinalbin/sinapin salt are derived from the same precursor, the C_6C_1 unit in sinapin should have the same δ ^{13}C-value as the corresponding molecule part of glucosinalbin, namely −33.2‰ (Table 1). From this value and the δ ^{13}C-value of the whole molecule sinapic acid (−32.5‰) a mean value of −29.6‰ can be calculated for its C-1/C-2-part (Fig. 1). Assuming that, as in phenylalanine and other compounds derived from pyruvate, the carboxyl group (C-1) is enriched relative to the rest of the molecule by at least 3.5‰, a δ ^{13}C-value of −33.1‰ can be calculated for the position C-2 of sinapic acid. This would confirm the depletion postulated for position C-2 of sinapic acid as a compensation for the enrichment of glucosinalbin in the corresponding position.

We suggest this indicates a possible carbon isotope effect on the PAL reaction. The mechanism of this reaction is well known, and the existence of nitrogen and hydrogen isotope effects are described for the positions C-2 and C-3, respectively [10]. This makes a ^{13}C-isotope effect on C-2 quite obvious, and should be measurable in a suitable *in vitro* system.

There is one unsolved problem with this theory: in many plants, tyrosine is a poor precursor of phenylpropanes [11], therefore the PAL reaction would not be of such importance as a competitor for the formation of cyanogenic glycosides and glucosinolates. However, according to our measurements (Table 2) the relative enrichment in position C-2 is identical for products from phenylalanine or tyrosine, and so we have to rely on the *in vitro* measurement of the kinetic isotope effect on the PDH-reaction for final verification.

(3) A third possible explanation for the ^{13}C-enrichment in position 2 of the compounds would be an isotope effect on one of the reactions involved in biosynthesis of the glucosinolates or cyanogenic glycosides from their amino acid precursors [12]. These involve *N*- and *C*-hydroxylations, followed by an irreversible decarboxylation. None of them would probably imply an isotope discrimination. It is, however, possible that the oxime, a common intermediate on the path to the nitrile or the thiohydroxamic acid, respectively, could be exposed to a competing hydrolysis [13], and that this reaction could imply an isotope effect. This explanation would demand a ^{13}C-enrichment in the corresponding position of glucosinolates and cyanogenic glycosides formed from other amino acids. However, this is not the case, at least for sinigrin. Hence the most probable explanation for the

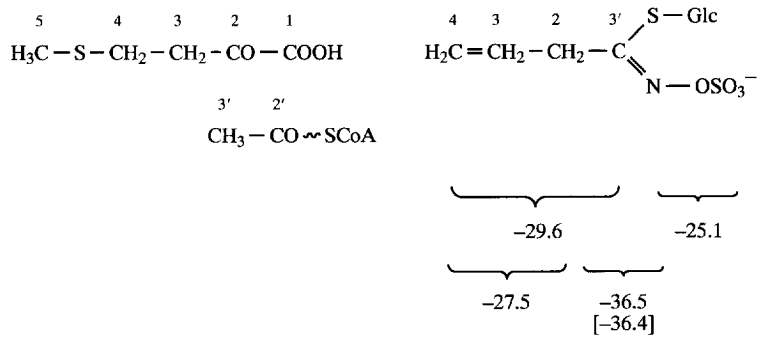


Fig. 3. δ ^{13}C-values $[‰]_{PDB}$ of sinigrin (−27.0), the glucosinolate from black mustard seed, and of fragments obtained by partial degradation ($n = 2$). Values in [ ] calculated from difference of allyl mustard oil (C-3′ to C-4) and allyl amine (C-2 to C-4). The atoms are numbered according to their precursor molecules acetyl-CoA and 2-oxo-4-(methylthio)-butyric acid (ketomethionine), which are condensed by an aldol reaction to form homomethionine. Positions C-1 to C-3 originally derive from pyruvate, C-3′ from position 3 of pyruvate.

^{13}C-enrichment in position C-2 of the aromatic glucosinolates and cyanogenic glycosides is an isotope effect on the PAL-reaction.

Partial isotope pattern of sinigrin

In order to verify whether our interpretation is generally valid, we studied the ^{13}C-pattern of sinigrin, the glucosinolate of *Brassica nigra*, the black mustard and *B. juncea*. As expected, the aglycone was distinctly depleted in ^{13}C relative to the sugar moiety (Fig. 3). However, the first C-atom of the mustard oil formed by hydrolysis of the glucosinolate, which corresponds to the α-C-atom of the precursor amino acid homomethionine, was remarkably depleted (Fig. 3). This constrasts greatly with the aromatic glucosinolates.

The biosynthesis of homomethionine (Fig. 4) proceeds from pyruvate via aspartate, homoserine and methionine to 2-oxo-4-(methylthio)-butyric acid (ketomethionin). This intermediate undergoes chain elongation, the key reaction of which is an aldol condensation with acetyl-CoA (Fig. 3). We have data indicating that this kind of reaction, analogous to the citrate synthase reaction, involves an isotope effect on the C-atom of the methyl group of acetyl-CoA, and prefers the 'light' molecules. Preliminary results with ^{13}C-NMR show also that C-2 of sinigrin like the corresponding position in citrate, is relatively enriched in ^{13}C (Bengsch, E. and Schmidt, H.-L., unpublished results).

Other reactions in the course of sinigrin biosynthesis with isotope effects could be responsible for this finding, especially as there are many branching points in this long biosynthetic pathway. However, we believe that for the sinigrin pattern an isotope effect on the hydrolysis of the intermediate oxime, as discussed for the phenylalanine derivatives, will probably not be the cause. Independent of the fact that products of such a hydrolysis are not found in *B. nigra*, an isotope effect on this step would not lead to a depletion but to an enrichment of the corresponding C atom of sinigrin. Therefore, this result also confirms the interpretation discussed for the pattern of the phenylalanine derivatives.

The relative depletion of position 3′ (C-1) in sinigrin has been found in all natural samples of allyl mustard oil analysed to date (Table 2). The data show that the carbon atom in position 1 is depleted by approximately 6‰ when compared with the whole isothiocyanate in all of the nine natural samples analysed. Conversely,

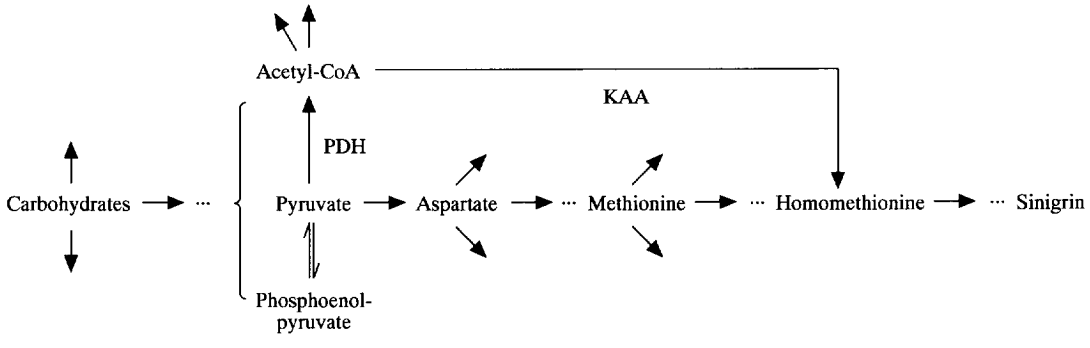


Fig. 4. Global pathways and branching points in the biosynthesis of the carbon skeleton of sinigrin. Enzyme-catalysed reactions with discussed isotope discriminations are indicated. PDH = pyruvate dehydrogenase (decarboxylating). KAA = α-ketoacid-acetyl-CoA-aldolase reaction.

Table 2. δ ^{13}C-values of allylisothiocyanates of different origin and of position C-1 in the molecule. Samples 1–3 were isolated from mustard seed; samples 4–9 were commercial, isolated from plant sources; samples 10 + 11 were synthetic compounds synthesized from CS_2 and allylamine, characterized by food laws as 'nature identical'

Sample no.	Origin	δ ^{13}C [‰]$_{PDB}$	
		Isothiocyante	Position C-1
1	*Brassica juncea* 1	−28.0	−36.2
2	*Brassica juncea* 2	−27.4	−33.3
3	*Brassica nigra* 1	−27.6	−31.1
4	Commercial natural 1	−28.2	−34.9
5	Commercial natural 2	−29.2	−37.4
6	Commercial natural 3	−29.0	−33.8
7	Commercial natural 4	−28.1	−35.8
8	Commercial natural 5	−28.0	−32.3
9	Commercial natural 6	−28.4	−35.2
	mean of samples 1–9	−28.2	−34.4
10	Commercial nature identical 1	−31.4	−45.1
11	Commercial nature identical 2	−29.4	−45.5

the synthetic products ('nature identical' in the sense of food laws) show a much larger depletion in this position (−14‰). This may be due to a pre-existing depletion in the starting material CS_2 or to an isotope effect on the first step of the reaction, in which allyl amine is condensed with CS_2 [14]. If the latter compound is in excess, as specified by the reaction conditions for the synthesis, the 'light' molecules would react preferably. Our results agree with those of Remaud [15], who earlier detected differences in the deuterium patterns of natural and synthetic allyl isothiocyanate, respectively, by means of site-specific NMR measurements.

DISCUSSION

As verified on many other occasions [1–3], secondary plant products are usually depleted in ^{13}C relative to their primary precursors, the carbohydrates. Obviously, amino acids and their derivatives, in the present case cyanogenic glycosides and glucosinolates, preserve partially in some positions the relative ^{13}C-pattern of their precursors. However, they also develop individual ^{13}C-abundances in other defined positions, owing to isotope effects on key reactions in their biosynthesis. These become evident in form of isotope discriminations, when the reactions are located at the metabolic branching points.

EXPERIMENTAL

Chemicals and enzymes. All ordinary chemicals were purchased in reagent grade from local sources. Thioglucosidase (E.C. 3.2.3.1.) was obtained from Sigma–Aldrich–Chemie, amygdalin was purchased from Roth, and recrystallized from EtOH. An enzyme preparation (emulsin) was obtained from the kernels of sweet almonds according to ref. [16]: 10 g sweet almond meal was defatted with petrol and then stirred with 50 ml H_2O at 20°. After 4 hr the suspension was filtered and the filtrate used as enzyme prepn.

Plant material and isolation of natural products. Bitter almond kernels (*Prunus amygdalus Batsch* var. *amara*), white mustard seeds (*S. alba*), black mustard seeds (*B. nigra*), seeds of *B. juncea* and seeds of garden cress (*Lepidium sativum*) were purchased from local markets or from other commercial sources. Leaves of the elder (*Sambucus nigra*) were collected from local orchards. Seeds of durra (*Sorghum sativum*) from a local shop were germinated in the dark, and the germs harvested as described in ref. [17]. Seeds of garden cress (*L. sativum*) were sown in trays with garden mould, and the plants, grown indoors, were harvested after six days.

Cellulose was extracted from plant material as described in ref. [18]. Amygdalin was extracted, purified and identified from the kernels of bitter almonds [19]. Sinalbin was obtained from white mustard seed as described in ref. [20]; yields and purity criteria were similar to those reported. Sinigrin was isolated from black mustard seed after ref. [21]. The mp of the compound was as lit. (145°). No impurities were detected by TLC (silica gel, *n*-BuOH–*n*-PrOH–HOAc–H_2O, 3:1:1:1, visualization with 1% $KMnO_4$ soln). The other glycosides were not isolated, but directly hydrolysed from the plant material.

Phenylalanine was isolated from the protein of bitter almonds according to ref. [22]: 100 g bitter almond meal was defatted with CH_2Cl_2 and air dried. The material was macerated in a 10% KCl solution, the mixt. was squeezed through a cloth and the filtrate centrifuged. From the clear soln, protein was prptd by adding $(NH_4)_2SO_4$ to 35% satn and isolated by centrifugation. After washing with deionized H_2O, it was redissolved in 10% KCl solution and dialysed overnight against deionized H_2O. The protein was prptd again, isolated by centrifugation and freeze-dried. Aliquots of 200 mg were hydrolysed in sealed flasks with 200 ml 6 N HCl at 110° for 24 hr. The hydrolysates were filtered and dried on a rotatory evaporator. The residue was repeatedly dissolved in deionized H_2O and evapd to eliminate the HCl. Phenylalanine was

sepd by ion-exchange chromatography on Amberlite (GC 120 II, 200–400 mesh, 15 × 300 mm) [23]; purity examined by PC (*n*-BuOH–HOAc–H_2O, 4:1:1).

Separation and degradation procedures

Cyanogenic glycosides. 1.1 g amygdalin was hydrolysed for 24 hr with 5 ml of the enzyme preparation from the kernels of sweet almonds [16] in 200 ml phosphate buffer (0.067 M, pH 5.8) at room temp. Then the mixt. was steam-distilled. From the distillate the benzaldehyde was extracted with Et_2O and distilled; its purity was checked by TLC and GC. In parallel experiments with 270 mg and 189 mg amygdalin, respectively, the benzaldehyde and HCN formed during hydrolysis was sepd from the reaction mixts by steam distillation into 1 N $AgNO_3$. The AgCN ppt. was isolated by filtration. Benzaldehyde and AgCN were submitted for combustion and isotope ratio MS.

The aq. soln remaining from the steam distillation of benzaldehyde was evapd to dryness under red. pres., and the residue was extracted ×4 with 50 ml EtOH to obtain glucose. The EtOH was removed, the residue was redissolved in H_2O and freeze-dried for isotopic analysis. Sambunigrin, the cyanogenic glycoside in leaves from elder, and dhurrin, the cyanogenic glycoside from sorghum seedlings, were hydrolysed directly in the plant material: 500 g were oven-dried (80°, 3 hr), ground to a fine powder and defatted with petrol. The air-dried material was suspended in 500 ml 0.067 M phosphate buffer pH 5.8, 10 g defatted meal of sweet almonds were added and the mixt was kept at room temp. for 24 hr. The benzaldehyde and the HCN formed were isolated for isotope analysis as described above.

p-Hydroxybenzaldehyde and HCN from 3-day-old sorghum seedlings (54 g) were isolated in a similar way. The aldehyde was purified by sublimation and analysed by TLC (*n*-BuOH–HOAc–H_2O, 4:5:1, upper layer, visualized by spray with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl). Both compounds were submitted to isotopic analysis.

Glucosinolates. The main constituents of sinalbin were isolated through independent incubations of the same starting material. The conversion of sinalbin to *p*-hydroxybenzyl isothiocyanate, glucose, choline and sinapic acid and the isolation of those compounds were accomplished by a modification of the method in ref. [24]: For the isolation of *p*-hydroxybenzyl isothiocyanate and sinapic acid, 250 mg sinalbin were hydrolyzed (37°, 5 hr) in 100 ml 0.5 N phosphate buffer pH 6.8 with thioglucosidase (0.25 μkat). The *p*-hydroxybenzyl isothiocyanate was extracted with CH_2Cl_2, characterized as the *N*-(*p*-hydroxybenzyl)-*N'*-*p*-phenylthiourea (mp 142°), and analysed for isotope abundance. The aq. phase containing sulphate, glucose and sinapin was made alkaline with $Ba(OH)_2$, and heated at 100° for 15 min. Then the solution was acidified with 2 N H_2SO_4 to approx. pH 2, and the $BaSO_4$ removed by centrifugation. The aq. phase containing glucose, sinapic acid and choline was concentrated *in vacuo* to 3 ml and chilled to 0°. This pptd sinapic acid which was isolated and recrystallized from MeOH–H_2O for isotope analysis. The aq. soln was discarded.

For the isolation of the glucose moiety 200 mg sinalbin were hydrolysed with thioglucosidase as described above. From the reaction mixt. *p*-hydroxybenzyl isothiocyanate was extracted with Et_2O and the aq. soln applied to an Amberlite CG 120 II column. Glucose was eluted with H_2O, identified with glucose oxidase, and the combined fractions were evapd to dryness. The remainder was used directly for isotope analysis.

For sepn of the main constituents and their partial degradation 300 mg sinalbin were dissolved in water and applied to an ion-exchange column (Amberlite CG 120 II, 200–400 mesh; 15 × 300 mm). Glucosinalbin was eluted with 80 ml water, and hydrolysed 1 hr with 10% HCl. The *p*-hydroxyphenylacetic acid formed was extracted with petrol, identified by TLC, and isolated for isotopic ratio MS. For the isotope analysis of the carboxyl group an aliquot was methoxylated [25], the derivative was decarboxylated as described in ref. [26], and the CO_2 (i.e. carbon 2, see Table 1) generated was analysed for its isotopic composition. The δ ^{13}C-value of glucosinalbin was calculated from those of *p*-hydroxyphenylacetic acid and glucose.

Benzyl cyanide, the hydrolysis product of glucotropeolin, was prepared from macerated garden cress seedlings (0.2 N phosphate buffer pH 3.2, 35°, 1 hr) and isolated by steam distillation. The product was extracted with Et_2O and distilled under red. pres. (bp_{13} 109°) and an aliquot used for isotope analysis. The main part was oxidized by heating for 4 hr in an excess of aq. alkaline $KMnO_4$ solution to 100°. The benzoic acid so formed was extracted with Et_2O and isolated for isotope analysis. The δ ^{13}C-value of C-2 was calculated from the difference of the δ ^{13}C-values of benzyl cyanide and benzoic acid.

A total of 200 mg sinigrin were hydrolysed (2 hr, 45°) in 100 ml phosphate buffer pH 6.8 and 80 nkat thioglucosidase to isolate the glucose. The allyl isothiocyanate was extracted with Et_2O, and the extract was discarded. The aq. soln was boiled, the denatured protein removed by centrifugation, and the clear solution transferred to an anion-exchanger (Dowex 1 × 8). The glucose was eluted with H_2O, and identified by glucose oxidase (62% yield). The frs containing glucose were collected, freeze-dried and submitted directly to isotope ratio analysis.

Allyl isothiocyanate was directly prepared from ground seeds of *B. nigra* or *B. juncea* according to ref. [27]. After digestion of the material in aqueous suspension for 1.5 hr at 45°, the mustard oil was volatilized by steam distillation, sepd from the aq. phase of the distillate and dried. The product was distilled in a bulb tube, and the purity examined by GC on Carbowax 20M. An aliquot was submitted for isotope analysis. The hydrolysis of the allyl isothiocyanate to allylamine and COS followed ref. [28]: a two-necked vessel with a nitrogen inlet was connected to a second two-necked round-bottomed flask, sealed with a tube with solid

NaOH, and containing alkaline $KMnO_4$ soln (400 mg $KMnO_4$ and 400 mg NaOH in 3 ml water), which was stirred by a magnetic stirrer. In the first two-necked vessel 200 mg allylisothiocyanate were heated with 1 ml 20% HCl to 110° for 15 min. Then the vessel was flushed with N_2, and the COS released was oxidized by the basic solution of $KMnO_4$ to yield carbonate. After centrifugation of the MnO_2 formed, the carbonate was precipitated as $BaCO_3$ (yield 25%). The acid solution containing allylamine hydrochloride was evapd until crystallization began, and the crystals were transferred into a bulb tube. A total of 0.2 ml 10 M KOH were added, and the allylamine liberated was distilled at 75°. The yield was 65%, and the compound was submitted to IR-MS. From the $BaCO_3$ CO_2 was released by HCl. This was purified by cryogenic techniques and transferred to the mass spectrometer. An alternative for the routine isotope analysis in position 1 was to heat a few μl of the mustard oil in a closed vessel with 1 ml 60% $HClO_4$ to 100° for 30 min. In this case, CO_2 is formed directly from the first C-atom (C-3′ in Fig. 3), the allyl amine was not isolated.

Phenylalanine was decarboxylated with ninhydrin [29]. The CO_2 from C-1 was collected in liquid nitrogen, and the phenylacetic acid formed was isolated by extraction and analysed. Independently, phenylalanine was oxidized in 1 N NaOH with an excess of $KMnO_4$ (100°, 8 hr) to benzoic acid. This compound was extracted, purified by sublimation, analysed (C_6C_1 unit) and decarboxylated in a sealed pyrex tube at 550° for 45 min (C-3). The CO_2 released was isolated from the reaction products on a vacuum line. The δ ^{13}C-value of position C-2 was calculated from the differences.

For isotope analysis the biological compounds and their degradation products isolated as described above were combusted in a closed system as described in ref. [30] and the carbon dioxide was transferred for isotope ratio analysis by MS (MM 903, VG Isogas, Middlewich, GB). The δ ^{13}C-values were measured against a laboratory standard and expressed relative to the PDB-standard.

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