Article

Biosynthetic Origin of the Isoprene Units in Chromenes of *Piper aduncum* (Piperaceae)

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Estudos metabólicos envolvendo a incorporação de *D*-glicose-[1-^{13}C] em folhas de *Piper aduncum* (Piperaceae) mostraram que as vias do mevalonato (MVA) e da triose-piruvato (MEP) estão envolvidas na biossíntese da unidade isoprênica presente no 2,2-dimetil-2*H*-1-cromeno-6-carboxilato de metila (**1**) e no 2,2-dimetil-8-(3'-metil-2'-butenil)-2*H*-1-cromeno-6-carboxilato de metila (**2**). O padrão de incorporação dos carbonos marcados com *D*-glicose-[1-^{13}C] destes cromenos foram determinados através de espectroscopia de RMN de ^{13}C quantitativo. Os resultados confirmaram que o compartimento biossintético de **1** e **2** pode ser o plastídio e/ou citosol, ou possivelmente um compartimento adicional, como o espaço entre a membrana e o plastídio.

Metabolic studies involving the incorporation of [1-^{13}C]-*D*-glucose into intact leaves of *Piper aduncum* (Piperaceae) have indicated that both the mevalonate (MVA) and the pyruvate-triose (MEP) non-mevalonate pathways are implicated in the biosynthesis of isoprene moieties present in methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) and methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**). The pattern of incorporation of label from [1-^{13}C]-*D*-glucose into these chromenes was determined by quantitative ^{13}C NMR spectroscopy. The results confirmed that biosynthetic compartment of **1** and **2** could either be the plastid and/ or the cytosol or, possibly, an additional compartment such as the plastid inter-membrane space.

Keywords: *Piper aduncum*, chromenes, [1-^{13}C]-*D*-glucose, mevalonate pathway, 2-*C*-methyl-*D*-erythritol-4-phosphate pathway

Introduction

The isoprenoids constitute a large class of natural products comprising more than 29,000 compounds.[1] In higher plants, isoprenoids play essential roles in membrane structure (sterols), redox chemistry (plastoquinones, ubiquinones), growth regulation (gibberellins, cytokinins, brassinosteroids and abscisic acid), defence mechanisms (phytoalexins) and free radical scavenging (carotenoids and tocopherols).[2] The common building blocks of all isoprenoids, the so-called 'isoprene units', is derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The biosynthesis of IPP can proceed *via* two different pathways, namely the long-established mevalonic acid (MVA) and the 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP) pathways, the reaction sequence of which has been characterized.[3] In plants, the MEP pathway appears generally operative for formation

of monoterpenoids, diterpenoids, phytols and carotenoids.[4-6] The key regulatory step of the MVA pathway involves reduction of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, with this being catalysed by the cytosolic enzyme HMG-CoA reductase (HMGR). Plastidic IPP is derived, however, from MEP formed *via* the condensation of pyruvate and glyceraldehyde-3-phosphate (G3P) catalysed by 1-deoxy-*D*-xylulose-5-phosphate synthase (DXS).[2,6] The two pathways to IPP are thus compartmentalized in the cytosol and the plastid, respectively. However, they may function in the formation of certain isoprenoids, in a cooperative process that results from metabolic cross-talk within the plastidic envelope membrane.[2]

Piper aduncum L. (Piperaceae) contains a number of chromenes that possess both anti-fungal and anti-tumour activities.[7,8] Recently, the prenylation of methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) to yield methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) has been reported.[9] In the present work, the

biosynthetic origins of the isoprene units present in **1** and **2**, isolated from the leaves of *P. aduncum*, have been determined from feeding experiments with [1-^{13}C]-*D*-glucose as (a putative) substrate. Metabolic studies involving this labelled precursor have previously been shown to provide a powerful strategy for the quantitative assessment of the differential contribution of the two isoprenoid pathways in the biosynthesis of individual terpenes.[10]

Experimental

Standards and chemicals

^{1}H and ^{13}C NMR spectra were recorded in CDCl$_3$ with a VARIAN INOVA 500 spectrometer. Chemical shift values were recorded relative to the δ_H (7.24 ppm) and δ_C (77.0 ppm) signals of the predominantly deuterated solvent. Silica gel (70-230 and 230-400 mesh, Merck) and C18 silica gel (Merck) were used for column chromatography. The [1-^{13}C]-*D*-glucose (99% isotopic abundance) was purchased from Sigma-Aldrich.

Plant material

Specimens of *Piper aduncum* L. were cultivated from seed under greenhouse conditions at the Institute of Chemistry, UNESP, Araraquara, SP, Brazil. Plant material was authenticated by Dr. Inês Cordeiro (Instituto de Botânica, São Paulo, SP, Brazil) and voucher specimen (Kato-57) has been deposited at the Herbário do Estado "Maria Eneyda P. Kaufmann Fidalgo" (São Paulo, SP, Brazil).

Administration of [1-^{13}C]-D-glucose into young leaves from P. aduncum

350 young leaves (210 g), freshly excised from 3-4 month old plants, were placed separately in 2 mL Eppendorf tubes and each administered 100 μL of a 0.1% solution of [1-^{13}C]-*D*-glucose in water at 25 °C for 72 h.[11,12]

Extraction and isolation

After incubation, the leaves were frozen in liquid N$_2$ and subsequentially extracted with EtOAc (2 × 25 mL), the solvent was removed by evaporation and the resulting crude extract (15.3 g) submitted to vacuum chromatography over silica gel (70-230 mesh) eluted with a hexane-EtOAc-MeOH in gradient elution. The EtOAc-soluble fraction obtained of the last fractionation was applied to a C18 silica gel column (Sorbent technologies-premium grade; 40-63 mm) and eluted with MeOH-H$_2$O (8:2) to yield five fractions (*A-*

E). Fraction *C* (0.120 g) was chromatographed twice over silica gel (230-400 mesh), eluting with hexane-acetone (9:1), to afford **1** (7.2 mg) and **2** (3.5 mg).

Spectroscopy

The ^{1}H and ^{13}C NMR spectra of samples of **1** and **2** obtained from incorporation experiments involving [1-^{13}C]-*D*-glucose as precursor or from field-grown plants (^{13}C at natural abundance) were measured under identical conditions. The relative abundances of ^{13}C at individual carbon atoms in **1** and **2** were calculated from the integrals obtained from the labelled sample in comparison with those of the natural abundance sample (Table 1). The values were normalised with reference to an abundance value of 1.1% for the carbon with the lowest ^{13}C enrichment. The ^{1}H and ^{13}C NMR data obtained were compared with 1D and 2D NMR data reported previously.[13,14]

Results and Discussion

The relative contributions of the two alternative biosynthetic routes to the 'isoprene units' may be readily deduced from the labelling patterns of the C5 units of isoprenoids synthesised from [1-^{13}C]-*D*-glucose. Label derived from [1-^{13}C]-*D*-glucose should be located at C-2 of acetyl CoA, and at C-3 of pyruvate and glyceraldehyde-3-phosphate. Thus DMAPP arising from three C-2 labelled acetyl CoA molecules *via* the MVA pathway should be labelled at C-2, C-4 and C-5. In contrast, DMAPP derived from C-3 labelled pyruvate and glyceraldehyde-3-phosphate *via* the MEP pathway should be labelled at C-1 and C-5.[15]

Leaves of *P. aduncum* were administered with [1-^{13}C]-*D*-glucose and incubated for 72 h. The leaves were harvested and extracted as described in the experimental section. The fractionation of the EtOAc lead to the isolation of methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) and methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**). The ^{13}C NMR spectra of **1** and **2** enriched with ^{13}C and that of a reference sample of **1** and **2** (^{13}C natural abundance) were measured in CDCl$_3$ under identical conditions, and the relative enrichment at each position was calculated by assuming an abundance of 1.1% for the carbon with the lowest ^{13}C-enrichment (Table 1).

The ^{13}C NMR spectrum of methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) biosynthesised from [1-^{13}C]-*D*-glucose showed significant enhancement of the signals at C-2', C-4' and C-5' (Table 1) indicating incorporation of label into the corresponding 'isoprene unit' *via* the MVA pathway as shown in Figure 1. In the pyran ring, however,

Table 1. Position-specific relative abundance of ^{13}C in methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) and methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) isolated from *Piper aduncum* following incorporation of [1-^{13}C]-*D*-glucose into intact leaves

Carbons	Chromene **1**		Chromene **2**	
	δ / (ppm)[a]	Relative enrichment / (%)[b]	δ / (ppm)[a]	Relative enrichment / (%)[b]
2	77.4	1.1	77.3	1.1
3	131.1	2.2	130.7	2.2
4	121.7	4.4	122.1	4.4
5	128.1	1.1	126.0	1.1
6	122.5	3.3	122.0	3.3
7	131.1	1.1	131.0	1.1
8	116.1	2.2	129.2	2.2
4a	120.6	2.2	120.4	2.2
8a	157.2	1.1	154.8	1.1
9 and 10	28.3	8.8	28.3	8.8
1'	-	-	28.2	1.1
2'	-	-	121.9	3.3
3'	-	-	132.4	1.1
4'	-	-	25.8	4.4
5'	-	-	17.9	2.2
OCH$_3$	51.8	4.4	51.7	4.4
C=O	166.8	1.1	167.1	1.1

[a]Chemical shift values referenced to CDCl$_3$. [b]Calculated from the relative intensities of signals in the ^{13}C NMR spectra of the respective labelled and natural abundance sample.

enrichment at positions C-4, C-9 and C-10 was observed corresponding, respectively, to C-1 and C-5 of IPP derived from the MEP pathway. In chromenes **1** and **2**, the two methyl groups C-9 and C-10 are enantiotopic and gave rise to a single ^{13}C-NMR signal, displaying a relative enrichment of 8.8 %. In this case, the data did not provide information for the precise distribution of

the label, but this did not interfere in the determination of the biosynthetic origin of the isoprene unit in pyran ring. The biosynthetic precursor of **2**, *i.e.* methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**), presented a similar enrichment pattern in the pyran ring (Figure 1). Thus, signals at C-4, C-9 and C-10 of **1** were enhanced (Table 1) in accord with incorporation of

Figure 1. Predicted labelling patterns for IPP and DMAPP units synthesised from [1-^{13}C]-*D*-glucose *via* the mevalonate pathway (MVA) and the non-mevalonate (MEP). The insert shows the experimentally determined positions of label in methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) and methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) isolated from *Piper aduncum* following incorporation of [1-^{13}C]-*D*-glucose into intact leaves. * enantiotopic groups.

[1-¹³C]-*D*-glucose *via* the MEP pathway. These data confirm that the initial prenylation of the *p*-hydroxybenzoic acid precursor of chromenes **1** and **2** occurred *via* MEP pathway (Figure 1). The aromatic and methoxyl carbons enrichments might be due to complex metabolic turnover of [1-¹³C]-*D*-glucose with following statistical distribution of the carbon atoms in various positions of intermediates of carbohydrate metabolism. These enrichments corroborated their biosynthesis in plants showing the same build blocks of the prenyl units derived from the glycolysis and the Krebs cycle that use the phosphoenolpyruvate, pyruvic acid and acetyl CoA as intermediates.

Conclusions

The present work is in accord with various studies that have suggested the simultaneous operation of two separate biosynthetic routes to the IPP/DMAPP units present in some plant isoprenoids.[5,12,16-23] The prenylation of the *p*-hydroxybenzoic acid involved in the formation of the chromenes **1** and **2** occurred via MEP pathway, suggesting that it is compartmentalized in the plastid. On the other hand, the second prenylation step would be occurred in the cytosol by MVA pathway. The observation of mixed biosynthesis, with contributions from both MVA and the MEP pathways, implies that certain biosynthetic steps proceed in different compartments, and that specific intermediates traverse the chloroplast boundary. At the enzymatic level, this hypothesis would imply the presence of two DMAPP synthases located in the plastid and in the cytosol. In *P. aduncum*, a prenyltransferase has already been identified.[9] This report constitutes the third example of the participation of DMAPP/IPP moieties derived from both MVA and MEP in the prenylation of benzoic acid derivatives.[11,12]

Acknowledgments

Supplementary Information

¹³C NMR spectra of **1** and **2** are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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Supplementary Information

Biosynthetic Origin of the Isoprene Units in Chromenes of *Piper aduncum* (Piperaceae)

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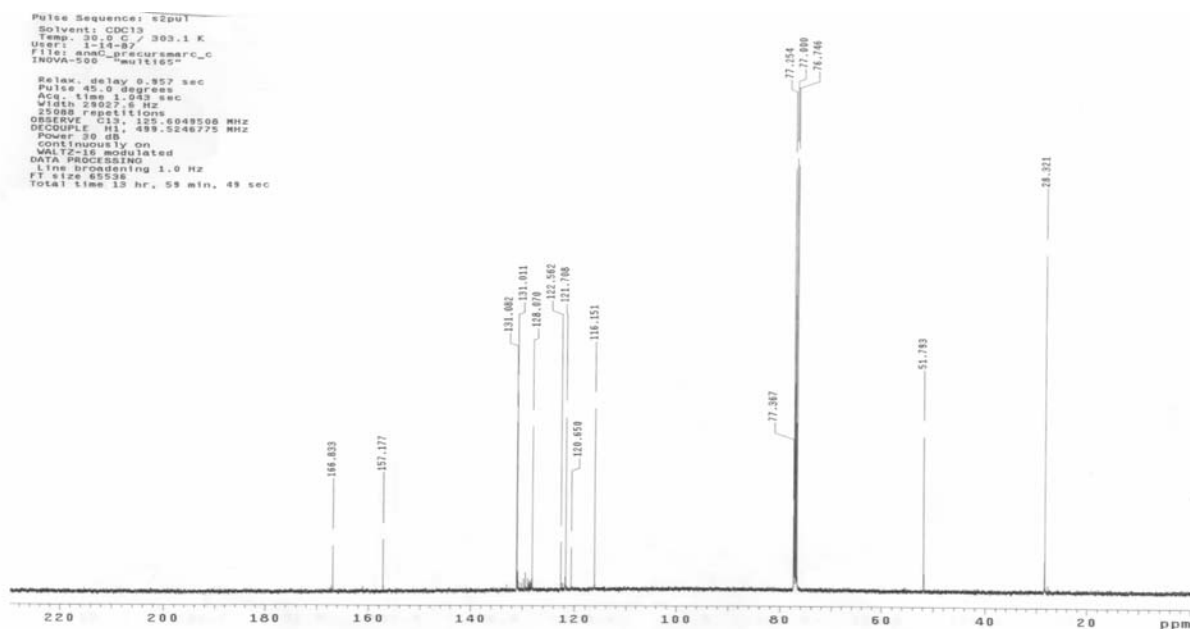*bInstituto de Química, Universidade de São Paulo, 05599-970 São Paulo-SP, Brazil*

Figure S1. ¹³C NMR spectrum of methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) (natural abundance sample, CDCl₃).

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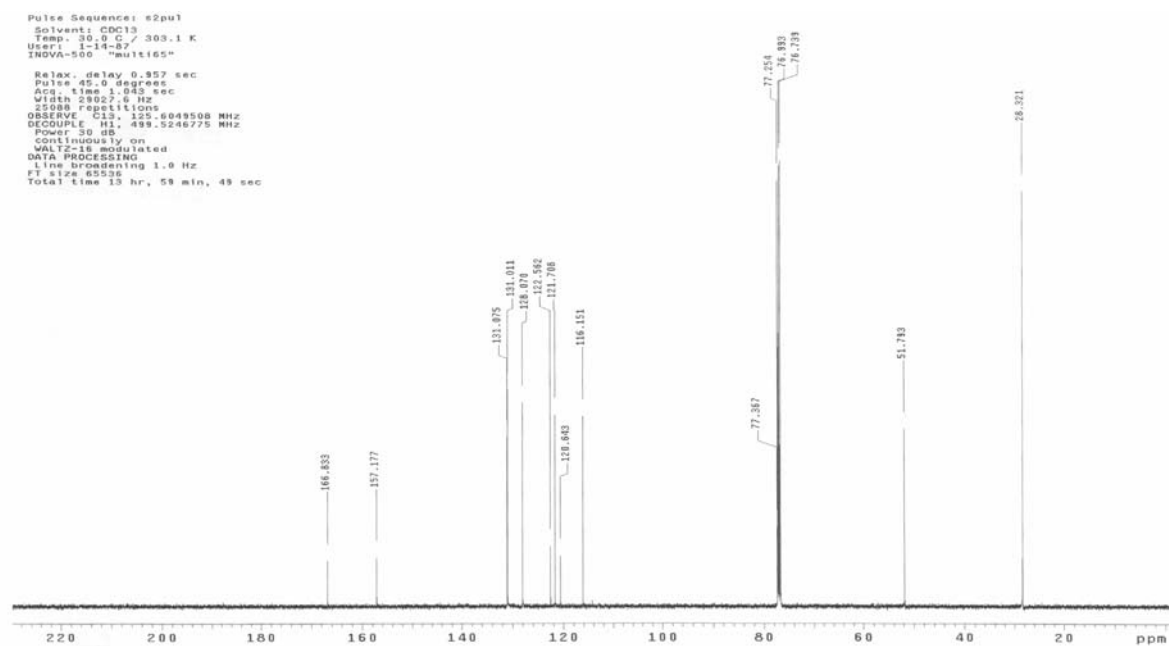
Figure S2. ¹³C NMR spectrum of methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**) (labelled sample, CDCl₃).
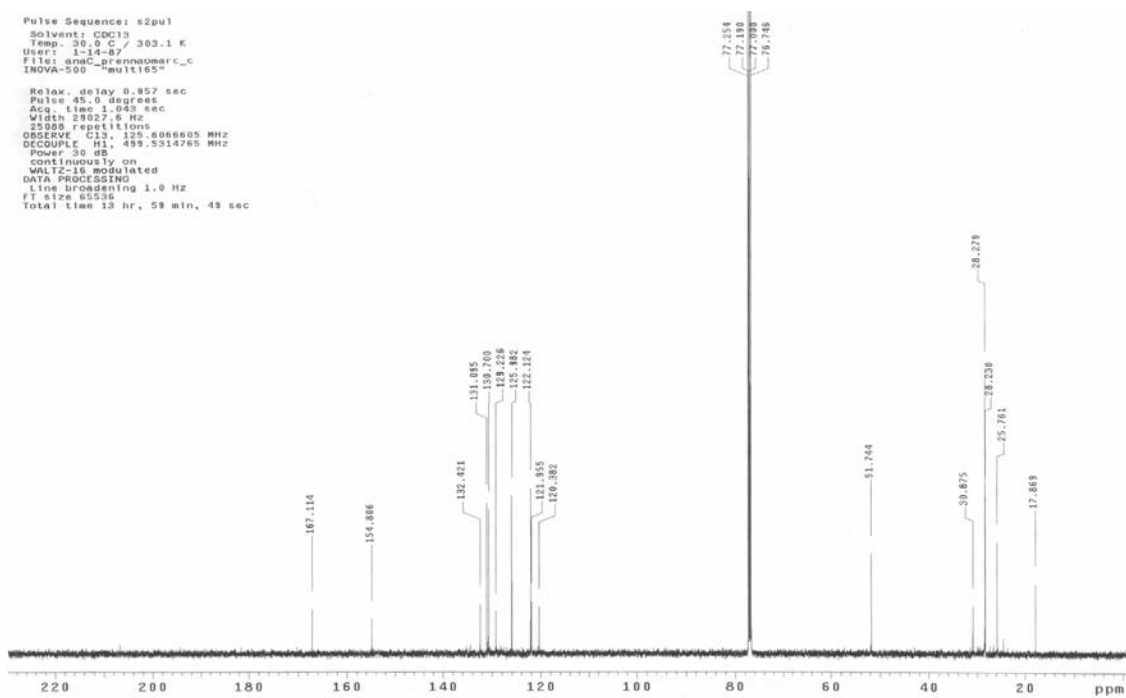

Figure S3. ¹³C NMR spectrum of methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) (natural abundance sample, CDCl₃).
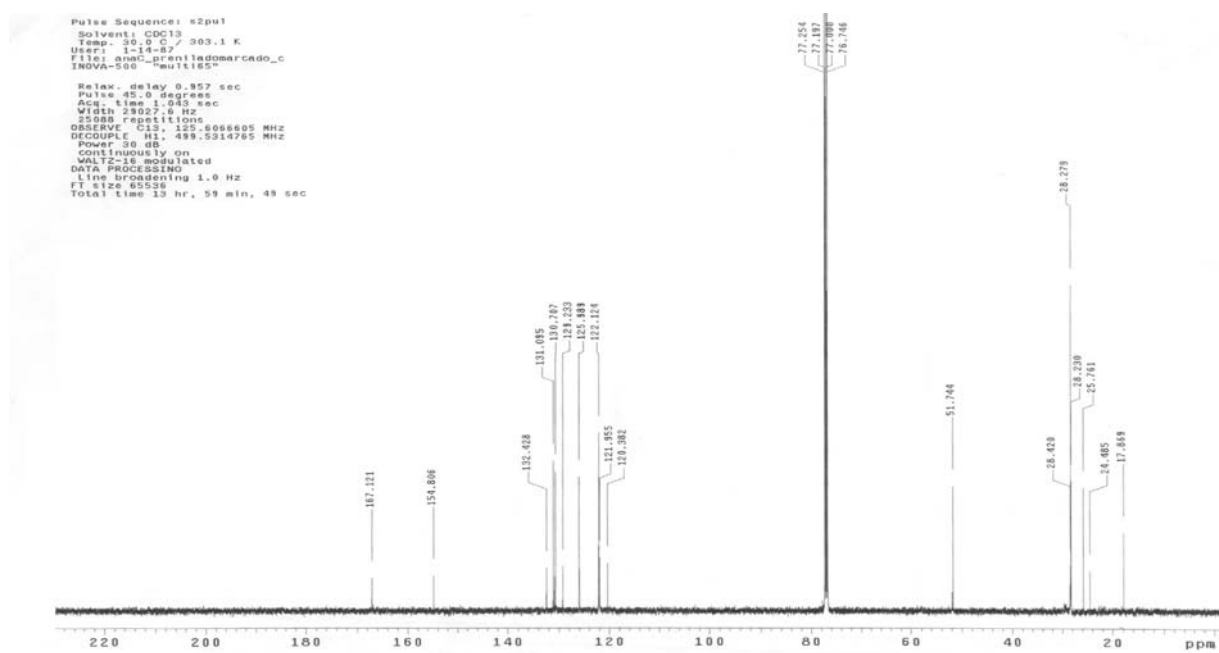
Figure S4. ^{13}C NMR spectrum of methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (**2**) (labelled sample, CDCl$_3$).