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Synthesis of dicarboxylic acylcarnitines

David W. Johnson*

Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Road, North Adelaide, South Australia 5006, Australia

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Abstract

Syntheses of malonyl, methylmalonyl, succinyl, glutaryl, methylglutaryl, dodecanedioyl and hexadecanedioyl carnitines are described. The dicarboxylic acylcarnitines were prepared from eight equivalents of cyclic anhydride or isopropylidene ester of the dicarboxylic acid and carnitine chloride in trifluoroacetic acid solution. Long chain dicarboxylic acylcarnitines were additionally purified by partitioning between water and *n*-butanol. Stable isotope labeled analogs, containing 3, 6 or 9 deuterium atoms, were also prepared. They are for use as standards in the electrospray ionization tandem mass spectrometric analysis of dicarboxylic acylcarnitines in samples from patients with inherited disorders of fatty acid oxidation.

Keywords: Dicarboxylic acid; Acylcarnitine; Glutarylcarnitine; Deuterium labeling; Tandem mass spectrometry; Newborn screening

1. Introduction

Newborn screening programs, utilizing tandem mass spectrometry, now aid in the diagnosis of many inherited diseases involving defects in fatty acid oxidation (Rashed, 2001; Chace et al., 2003). These diseases are characterized by abnormally high levels of acylcarnitines of fatty acids. Monocarboxylic acylcarnitines are measured using isotope labeled internal standards. The standards are synthesized from acyl chlorides and carnitine chloride (Ziegler et al., 1966; Bohmer and Bremer, 1968). Dicarboxylic acylcarnitines, which are diagnostic metabolites for the inherited diseases summarized in Table 1, are measured relative to the labeled monocarboxylic acylcarnitine with the closest mass. This is a consequence of the lack of commercially available dicarboxylic acylcarnitines and isotope labeled analogs. It results in poor accuracy and precision in the measurement of dicarboxylic acylcarnitines, especially glutarylcarnitine, which are typically found at lower concentrations than most monocarboxylic acylcarnitines.

Dicarboxylic acylcarnitine standards would also be of value in the differential diagnosis of dicarboxylic aciduria in urine samples (Shimizu et al., 1994) collected for organic aciduria screening.

The synthesis of succinylcarnitine (C4DC, conventional nomenclature for an acyl carnitine of a four carbon dicarboxylic acid) from succinyl chloride has previously been shown (Bohmer and Bremer, 1968) to afford a product with only 70% purity. The major problem in coupling a dicarboxylic acid to carnitine is that one carboxyl group must be protected during the esterification to avoid a binary derivative. Subsequent deprotection reactions result in solvolysis of the acylcarnitine ester. A synthetic strategy that effectively unmasks a carboxylic acid group during the esterification

* Fax: +61-8-81617100.
E-mail address: david.johnson@adelaide.edu.au (D.W. Johnson).

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Table 1
Dicarboxylic acylcarnitines and the inherited diseases for which they are diagnostic metabolites

Acylcarnitine	Inherited disease	Reference
C3DC	Malonic aciduria	Matalon et al. (1993)
	Malonyl CoA decarboxylase deficiency	Santer et al. (2003)
C4*br*DC	Methylmalonic aciduria	Oberholzer et al. (1967)
C5DC	Glutaric aciduria type I	Baric et al. (1998)
C6*br*DC	HMG-CoA lyase deficiency	Roe et al. (1986)
C12DC	Reye's syndrome	Tracey et al. (1988)
C12DC	Carnitine palmitoyltransferase type II	Fontaine et al. (1998)
C12DC	Peroxisomal biogenesis defect	Rocchicioli et al. (1996)
C14DC	Peroxisomal biogenesis defect	Rocchicioli et al. (1996)
C16DC	Peroxisomal biogenesis defect	Rizzo et al. (2003)
	Carnitine-acylcarnitine translocase deficiency	Roschinger et al. (2000)
C18DC	Peroxisomal biogenesis defect	Rizzo et al. (2003)
	Carnitine-acylcarnitine translocase deficiency	Roschinger et al. (2000)

br indicates a branched dicarboxylic acid.

of an alcohol, is the use of an anhydride. There are no previous reports of the use of anhydrides to prepare carnitine esters.

The synthesis of a comprehensive selection of dicarboxylic acylcarnitines, outlined in Scheme 1, was accomplished using this strategy. In addition, analogs of dicarboxylic acylcarnitines that are deuterium labeled in either the acyl group or the carnitine were prepared. They will be used for the quantification of dicarboxylic acylcarnitines by electrospray ionization tandem mass spectrometry (ESI-MS/MS).

2. Experimental

2.1. Chemicals

(Methyl-$^2H_3$) L-carnitine chloride (enrichment >99% $^2H_3$), (methyl-$^2H_9$)-DL-carnitine chloride (enrichment 99.8% $^2H_9$) and $^2H_6$-pentanedioic acid (enrichment 98.3% $^2H_6$) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada).

Glutaric anhydride (97% purity) was purchased from Lancaster (Eastgate, Lancashire, UK). All other chemicals and solvents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2. Mass spectrometric analysis

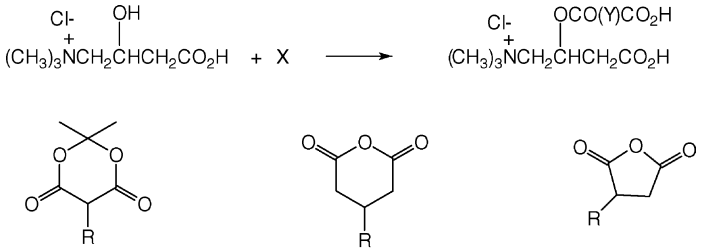
Mass spectrometric analysis was performed on an Applied Biosystems/SCIEX API365 tandem mass spectrometer (Concord, Ontario, Canada) fitted with an Ionspray assembly and operated in positive ion mode. Samples for analysis were dissolved in acetonitrile/water/formic acid (50:50:0.025, v/v/v) and infused, at 10 μl/min, with a Harvard syringe pump (Cambridge, MA, USA).

2.3. NMR analysis

NMR spectra were obtained on a Varian Inova 600 NMR spectrometer (Palo Alto CA, USA) operated by Mr. Philip Clements in the Adelaide University Department of Chemistry.

2.4. Preparation of DL-glutarylcarnitine chloride C5DC (3)

DL-Carnitine chloride (200 mg), glutaric anhydride (900 mg) and trifluoroacetic acid (0.35 ml) were heated at 75 °C, in a sealed tube, for 18 h. Acetone (4 ml) was added to the cooled mixture. Diethyl ether (5 ml) was then added dropwise over 15 min. The mixture was centrifuged (4000 rpm), the supernatant discarded, and the residue was washed twice with diethyl ether. The residue was dissolved in methanol (0.3 ml), again precipitated from acetone/diethyl ether, washed twice with diethyl ether and dried thoroughly in a stream of nitrogen. DL-Glutarylcarnitine chloride **3** (300 mg, 95% yield) was obtained as a colourless gum. It resisted all attempts at crystallization. ^1H NMR (D_2O, 600MHz): δ 1.88 (m, 2H, $CH_2CH_2CH_2$), 2.41



X_A X_B X_C

Compound no.		X	R	Y
1	C3DC	X_A	H	- CH_2 -
2	C4*br*DC	X_A	CH_3	- $CH(CH_3)$ -
3	C5DC	X_B	H	- $CH_2CH_2CH_2$ -
4	C6*br*DC	X_B	CH_3	- $CH_2CH(CH_3)CH_2$ -
5	C4DC	X_C	H	- CH_2CH_2 -
6	C12*br*DC	X_C	C_8H_{17}	- $CH_2CH(C_8H_{17})$ -
7	C16*br*DC	X_C	$C_{12}H_{25}$	- $CH_2CH(C_{12}H_{25})$ -

Scheme 1. Synthesis of dicarboxylic acylcarnitines from carnitine chloride and anhydrides and isopropylidene esters of dicarboxylic acids.

(m, 2H, $CH_2CH_2CO_2H$), 2.50 (m, $OCOCH_2CH_2$), 2.78 (m, 2H, $CHCH_2CO_2H$), 3.16 (m, 9H, $N(CH_3)_3$), 3.76 (m, 2H, NCH_2CH), 5.65 (m, ^1H, CH_2CHCH_2). Purity was determined as 96% from trimethylamine protons. ESI mass spectrum (see Fig. 1A): *m/z* 276 $(M)^+$, 217 $(M - N(CH_3)_3)^+$, 199 $(M - N(CH_3)_3, H_2O)^+$, 144 $(M - CH_2(CH_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 388 $(M)^+$.

2.5. Preparation of $^2H_6$-DL-glutarylcarnitine chloride d6C5DC

$^2H_6$-Pentanedioic acid (800 mg) and acetic anhydride (10 ml) were heated at 75 °C for 18 h. The mixture was evaporated in a stream of nitrogen. Two (5 ml) portions of dichloromethane were added and evaporated to remove the last traces of acetic acid. The $^2H_6$-glutaric anhydride, which was obtained as white crystals, m.p. 60 °C, was reacted with DL-carnitine chloride (150 mg) in the same manner described above to afford $^2H_6$-DL-glutarylcarnitine (175 mg, 73% yield) as a colourless gum. ^1H NMR (D_2O, 600 MHz): δ 2.78 (m, 2H, $CHCH_2CO_2H$), 3.16 (m, 9H, $N(CH_3)_3$), 3.76 (m, 2H, NCH_2CH), 5.65 (m, ^1H, CH_2CHCH_2). Purity was determined as 92% from trimethylamine protons. ESI mass spectrum (see Fig. 1B): *m/z* 282 $(M)^+$, 223$(M - N(CH_3)_3)^+$, 205 $(M - N(CH_3)_3, H_2O)^+$, 144 $(M - C^2H_2(C^2H_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 394 $(M)^+$.

2.6. Preparation of DL-methylglutarylcarnitine chloride C6brDC (4)

DL-Methylglutarylcarnitine chloride **4** was prepared, as a colourless gum, in an identical manner (91% yield) to that described for DL-glutarylcarnitine

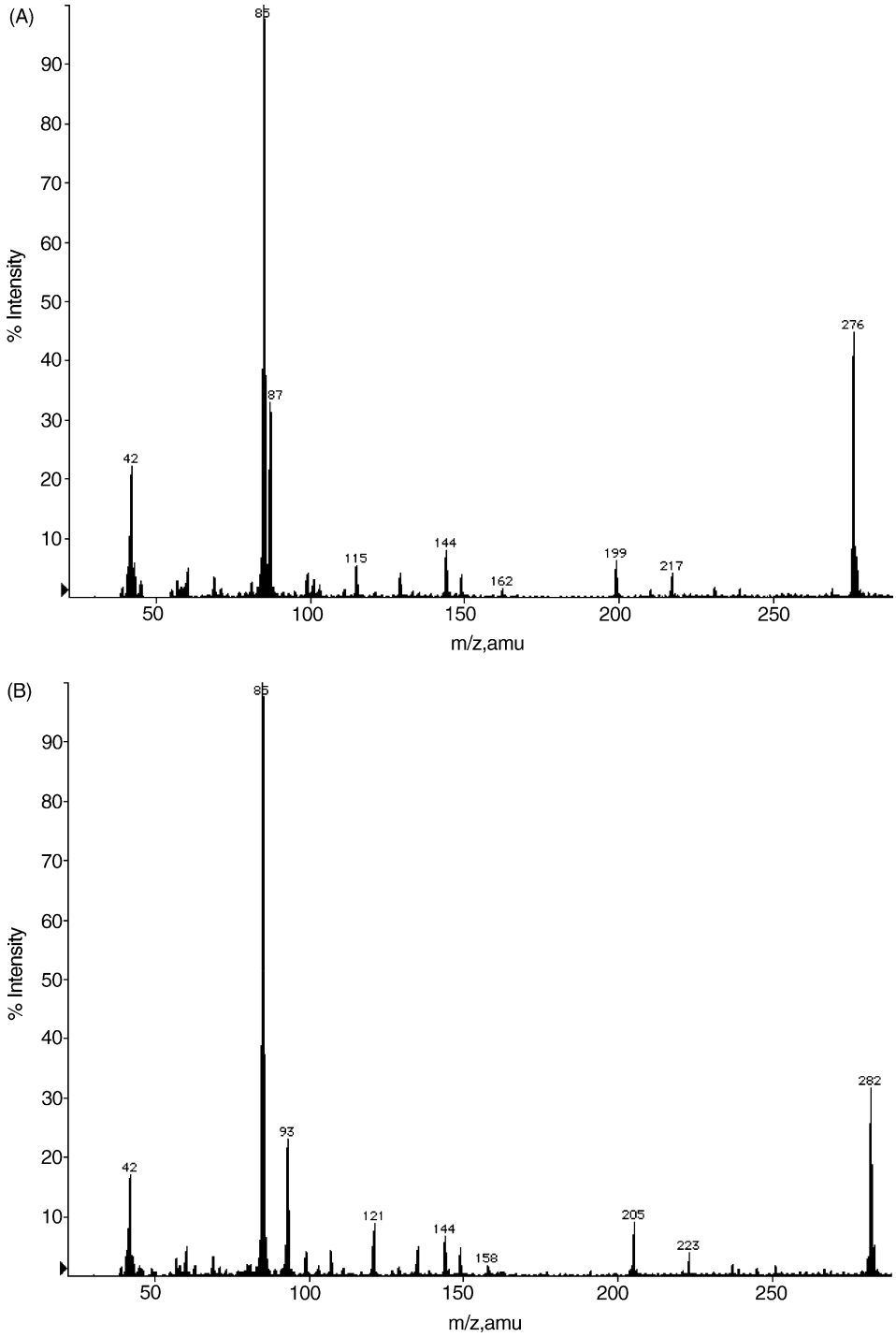


Fig. 1. ESI mass spectra of (A) C5DC and (B) $^{2}H_{6}$-C5DC.

chloride by substituting 3-methylglutaric anhydride for glutaric anhydride. Purity was determined as 90% by mass spectrometry. ESI mass spectrum: *m/z* 290 $(M)^+$, 231 $(M - N(CH_3)_3)^+$, 213 $(M - N(CH_3)_3, H_2O)^+$, 144 $(M - CH(CH_3)(CH_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 402 $(M)^+$.

2.7. Preparation of $^2H_3$-L-methylglutarylcarnitine chloride d3C6BrDC

$^2H_3$-L-Methylglutarylcarnitine was prepared, in 90% purity, from 3-methylglutaric anhydride and (methyl-$^2H_3$) L-carnitine chloride. ESI mass spectrum: *m/z* 293 $(M)^+$, 231 $(M - N(CH_3)_2(C^2H_3))^+$, 213 $(M - N(CH_3)_2(C^2H_3), H_2O)^+$, 147 $(M - CH(CH_3)(CH_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 405 $(M)^+$.

2.8. Preparation of DL-succinylcarnitine chloride C4DC (5)

A mixture of powdered succinic anhydride (790 mg) and DL-carnitine chloride (200 mg) was heated in a sealed container, under a nitrogen atmosphere, at 130 °C for 16 h. The cooled mixture was washed repeatedly with acetone. The remaining gum was dissolved in methanol and twice precipitated from acetone (4 ml) and diethyl ether (6 ml). DL-Succinylcarnitine chloride **5** (200 mg, 66% yield) was obtained as a pale brown, viscous oil of approximately 85% purity by mass spectrometry. ESI mass spectrum: *m/z* 262 $(M)^+$, 203 $(M - N(CH_3)_3)^+$, 144 $(M - (CH_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 374 $(M)^+$.

2.9. Preparation of DL-malonylcarnitine chloride C3DC (1)

2,2-Dimethyl-1,3-dioxane-4,6-dione (500 mg), DL-carnitine chloride (100 mg) and trifluoroacetic acid (0.2 ml) were heated at 45 °C for 18 h. Acetone (2 ml) and then diethyl ether (8 ml) were added. The mixture was centrifuged (4000 rpm) and the supernatant discarded. The red oil was washed twice with diethyl ether and dissolved in methanol (1 ml). The methanol solution was passed through a column (4 mm diameter × 40 mm) of Florisil in a Pasteur pipette to remove some colouration. Evaporation afforded DL-malonylcarnitine chloride **1** (150 mg, 88% yield) as a yellow, viscous oil. Purity was determined as 90% by mass spectrometry. ESI mass spectrum: *m/z* 248 $(M)^+$, 162 $(M\text{-}O{=}C{=}CHCO_2H)^+$, 144 $(M\text{-}CH_2(CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 360 $(M)^+$.

2.10. Preparation of DL-methylmalonylcarnitine chloride C4brDC (2)

DL-Methylmalonylcarnitine chloride **2** was prepared in an identical manner as described for DL-Glutarylcarnitine chloride by substituting 2,2,5-trimethyl-1,3-dioxane-4,6-dione for glutaric anhydride. It was obtained as a colourless gum (61% yield). Purity was determined as 90% by NMR. ^1H NMR (D_2O, 600 MHz): δ 1.38 (s, 3H, $CHC\mathit{H}_3$), 2.84 (m, 2H, $CHC\mathit{H}_2CO_2H$), 3.19 (m, 9H, $N(C\mathit{H}_3)_3$), 3.81 (m, 2H, $NC\mathit{H}_2CH$), 5.70 (m, ^1H, $CH_2C\mathit{H}CH_2$). ESI mass spectrum: *m/z* 262 $(M)^+$, 203 $(M - N(CH_3)_3)^+$, 185 $(M - N(CH_3)_3, H_2O)^+$, 144 $(M - CH(CH_3)(CH_2CO_2H)_2)^+$. ESI mass spectrum of dibutyl ester: *m/z* 374 $(M)^+$.

2.11. Preparation of DL-octan-1-ylsuccinylcarnitine chloride C12brDC (6)

2-Octen-1-ylsuccinic anhydride (5.0 g), DL-carnitine chloride (600 mg) and trifluoroacetic acid (1.0 ml) were heated at 65 °C for 18 h. The cooled mixture was diluted with dichloromethane (5 ml) and evaporated in a stream of nitrogen to remove most of the trifluoroacetic acid. The residue was dissolved in diethyl ether (5 ml) and extracted with water (2 × 2 ml). The aqueous solution was washed with diethyl ether (2 × 2 ml) and then extracted with *n*-butanol (3 × 3 ml, saturated with water). The combined *n*-butanol solution was washed with water (2 × 1 ml). The combined aqueous layers were again extracted with *n*-butanol (3 × 3 ml, saturated with water) and washed with water (2 × 1 ml). Evaporation of the combined *n*-butanol solutions afforded DL-2-octen-1-ylsuccinylcarnitine chloride (103 mg) as a colourless oil. The DL-2-octen-1-ylsuccinylcarnitine chloride was dissolved in ethanol (3 ml) and palladium on carbon catalyst (20 mg, 10% w/w) was added. The mixture was stirred under an atmosphere of hydrogen for 4 h, filtered through Celite, and evaporated

to dryness. DL-Octan-1-ylsuccinylcarnitine chloride **6** was obtained as a colourless gum (90 mg, 7% overall yield). Better yields were subsequently obtained on a smaller scale preparation with the same solvent purification volumes. It contained approximately 2% free carnitine. ESI mass spectrum: *m*/*z* 374 $(M)^+$, 315 $(M - N(CH_3)_3)^+$. ESI mass spectrum of dibutyl ester: *m*/*z* 486 $(M)^+$.

2.12. Preparation of DL-dodecan-1-ylsuccinylcarnitine chloride C16brDC (7)

The above reaction was repeated using 2-dodecen-1-ylsuccinic anhydride. DL-Dodecan-1-ylsuccinylcarnitine chloride **7** was obtained as a colourless gum in 7% overall yield. It contained approximately 2% free carnitine. ESI mass spectrum: *m*/*z* 430 $(M)^+$, 371 $(M - N(CH_3)_3)^+$. ESI mass spectrum of dibutyl ester: *m*/*z* 542 $(M)^+$.

2.13. Preparation of $^2H_9$-DL-octan-1-ylsuccinylcarnitine d9C12brDC and $^2H_9$-DL-dodecan-1-ylsuccinylcarnitine d9C16brDC chlorides

These two deuterium labeled dicarboxylic acylcarnitines were prepared from (methyl-$^2H_9$) DL-carnitine chloride and 2-octen-1-ylsuccinic and 2-dodecen-1-ylsuccinic anhydrides. Overall yields of 42 and 35% respectively, were obtained using 100 mg of $^2H_9$-DL-carnitine chloride. They contained <2% free carnitine. ESI mass spectra: *m*/*z* 383 $(M)^+$ and 439 $(M)^+$, respectively. ESI mass spectra of dibutyl esters: *m*/*z* 495 $(M)^+$ and 551 $(M)^+$, respectively.

3. Results and discussion

Eight equivalents of glutaric anhydride were reacted with carnitine chloride, in trifluoroacetic acid at 65 °C. Thin layer chromatography on silica with chloroform/methanol/concentrated ammonia solution (25:15:4, v/v/v) and staining with iodine revealed only the C5DC (rf 0.4) and a small amount of carnitine (rf 0.05). An ESI mass spectrum showed C5DC and carnitine in the ratio 34:1. ^1H NMR analysis showed the C5DC to be 92% pure (from the ratio of trimethylamine proton peaks). With a reaction temperature of 75 °C the purity improved to 96%. C5DC was always obtained as a gum despite numerous attempts at crystallization. It is both hygroscopic and absorptive of solvent of crystallization. Attempts to remove the small amount of unreacted carnitine were counterproductive since the polar solvents, like methanol and water, necessary to dissolve the acylcarnitine, slowly solvolysed it. For the preparation of a deuterium labeled analog of C5DC, $^2H_6$-pentanedioic acid was dehydrated to $^2H_6$-glutaric anhydride and reacted with carnitine chloride. The ESI mass spectra of C5DC and $^2H_6$-C5DC are shown, for comparison, in Fig. 1. Unreacted carnitine can be seen as the small ion with *m*/*z* 162.

ESI-MS/MS analysis, with a precursor ion (85 Da) scan, of an equimolar mixture of the butyl esters of $^2H_3$-decanoylcarnitine and $^2H_6$-C5DC afforded ions at *m*/*z* 375 and 394, in the ratio 2.7:1. This confirms that if $^2H_3$-decanoylcarnitine (the closest in mass to C5DC) was used as a standard to measure C5DC, without correction, the true amount of C5DC would be 2.7 times higher. This is in agreement with comparisons of C5DC and $^2H_3$-octanoylcarnitine (Chace et al., 2003).

Methylglutarylcarnitine (C6*br*DC, where *br* refers to a branched dicarboxylic acid) was similarly prepared from 3-methylglutaric anhydride. Since no equivalent deuterium labeled methylpentanedioic acid was available, $^2H_3$-C6*br*DC was prepared from $^2H_3$-carnitine chloride.

Succinylcarnitine (C4DC) required a modified experimental procedure. Because succinic anhydride is almost insoluble in trifluoroacetic acid, a powdered mixture of succinic anhydride and carnitine chloride was heated at just above its melting point. An unavoidable minor contamination (approximately 10%) was crotonic acid betaine (dehydrated carnitine) from thermal decomposition of the carnitine ester. Crotonic acid betaine was difficult to initially identify by ESI-MS/MS analysis because it has the same mass as a fragmentation ion (with *m*/*z* 144) of C4DC. It afforded a more prominent ion, with *m*/*z* 200 (203 for $^2H_3$ analog), in the ESI mass spectrum of the product after butylation.

The successful syntheses of C6*br*DC and C4DC prompted a modified strategy for preparing larger dicarboxylic acylcarnitines. Cyclic anhydrides with a ring size >6 atoms are not commercially available. 2-Substituted succinic anhydrides with various length alkenyl side chains, however, can be purchased. Dicarboxylic acylcarnitines prepared from them would

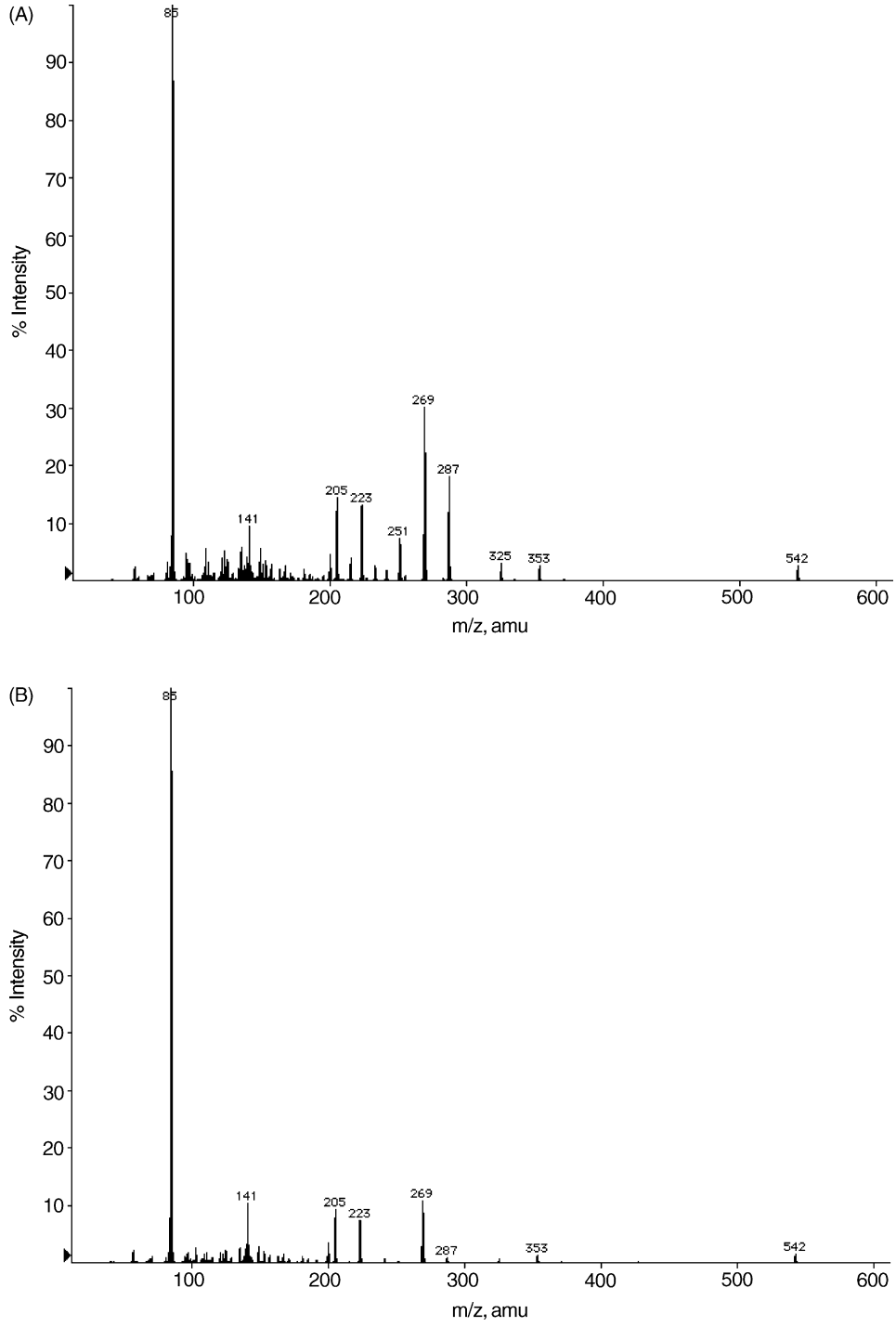


Fig. 2. ESI-MS/MS analysis by a product ion scan of the dibutyl ester of (A) C16DC and (B) C16*br*DC.

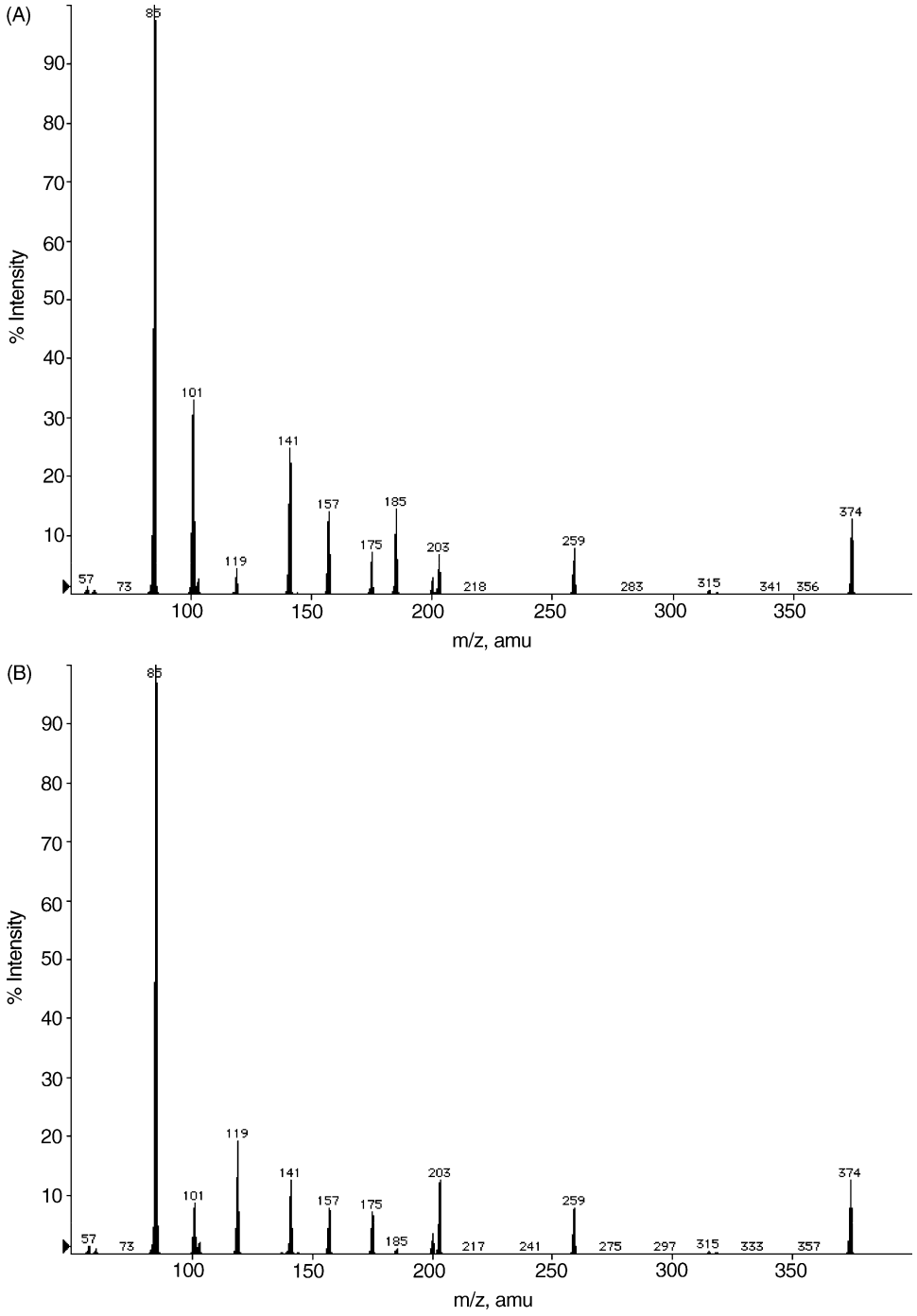


Fig. 3. ESI-MS/MS analysis by a product ion scan of the dibutyl ester of (A) C4DC and (B) C4*br*DC.

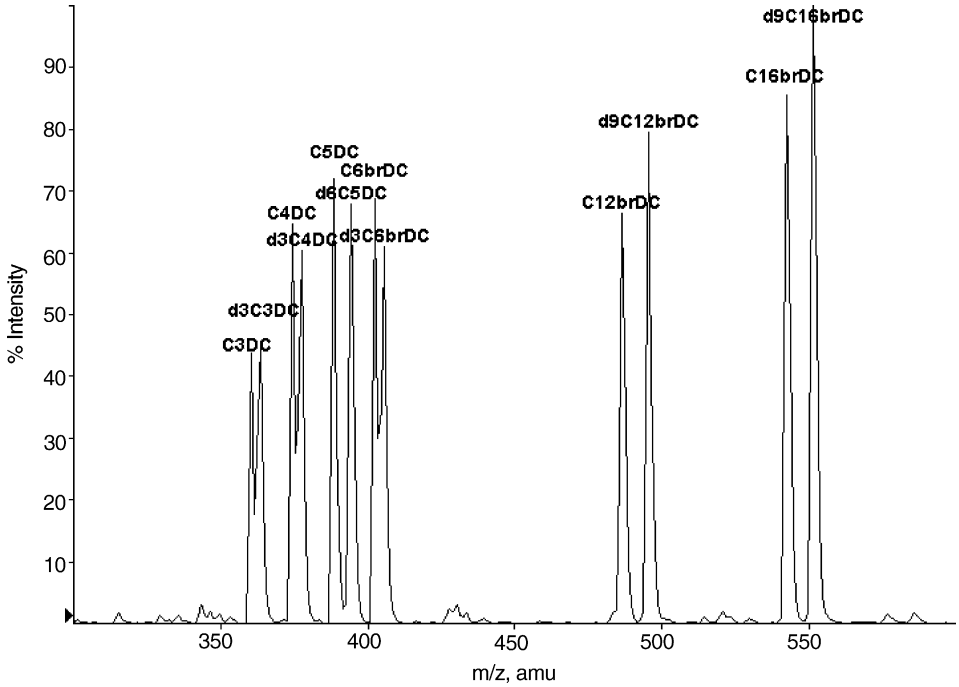


Fig. 4. ESI-MS/MS analysis by a precursor ion scan (85 Da) of an equimolar mixture of butyl esters of synthesised dicarboxylic acylcarnitines and deuterium labeled analogs.

be isomeric with those formed biologically (from straight chain dicarboxylic acids). Dicarboxylic C12 (dodecanedioyl), C16 (hexadecanedioyl) and C18 (octadecanedioyl) carnitine esters were prepared by this strategy. Eight equivalents of 2-substituted C8:1, C12:1 and C14:1 succinic anhydrides esterified approximately 60%, 30% and <10%, respectively of the carnitine at 65 °C. Increasing the temperature above 75 °C improved the esterification but afforded crotonic acid betaine (dehydrated carnitine) from thermolysis of the carnitine ester. Consequently, the partially esterified mixture was purified by extraction from aqueous solution into *n*-butanol, as used in the extraction of acylcarnitines from urine (Morrow and Rose, 1992). The double bond was removed by catalytic hydrogenation. The ESI mass spectrum showed that the resultant dicarboxylic acylcarnitines contained less than 2% carnitine. Insufficient C18*br*DC was obtained for it to be completely characterised. $^{2}H_{9}$-labeled analogs of C12*br*DC and C16*br*DC were prepared from $^{2}H_{9}$-carnitine chloride.

A small amount of *n*-hexadecanedioylcarnitine (C16DC) chloride was prepared from hexadecanedioic acid, via its acid chloride, in the manner used for monocarboxylic acylcarnitines. Although this was impure a clean ESI-MS/MS product ion spectrum could be generated from the molecular cation of its butyl ester. Comparison of the product ion spectrum with that of the butyl ester of C16*br*DC is shown in Fig. 2. The C16*br*DC showed fewer higher mass product ions relative to the product ion with 85 Da. This affords an estimated 10% greater response for ESI-MS/MS quantification of C16*br*DC using an 85 Da precursor ion scan.

Preparation of malonylcarnitine (C3DC) involved another variation of the strategy. Eight equivalents of Meldrum's acid (the isopropylidene ester of malonic acid) were used, instead of an anhydride, in the reaction with carnitine chloride. Meldrum's acid is a reactive substrate and rapidly turns a deep red colour in trifluoroacetic acid. The C3DC, even after chromatography from Florisil, is an oily substance that retains a yellow colour. ESI-MS analysis reveals a purity of approximately 90%.

The preparation of methylmalonylcarnitine (C4*br*DC) required a higher temperature (75 °C) but af-

forded a colourless product. The ^{1}H NMR indicated a minor contaminant (6%) with trimethylamine protons in addition to unreacted carnitine (4%). The ESI mass spectrum of C4*br*DC after butylation showed no unaccounted for ions. The ESI mass spectrum of underivatized C4*br*DC, however, contained an ion at *m*/*z* 218, which corresponds to loss of CO_2 from C4*br*DC. The identity of this contaminant has not been established. The ESI-MS/MS product ion spectra of the molecular cations of the butyl esters of C4DC and C4*br*DC are shown in Fig. 3. The two isomers can be differentiated by the relative intensities of ions at *m*/*z* 119 and 185. This observation may be of potential use in differentiating a patient with methylmalonic aciduria or methyl-CoA-decarboxylase deficiency from one with generalized dicarboxylic aciduria. In principle, it should be possible to separately measure C4DC and C4*br*DC in a mixture using the strategy developed for the butyl esters of methylmalonic and succinic acids (Kushnir et al., 2001). Likewise, differences in the product ion spectra of C6DC and C6*br*DC could also be exploited.

An equimolar mixture of C3DC, C4DC, C5DC, C6*br*DC, C12*br*DC and C16*br*DC and a deuterium labeled analog of each was butylated. The butylation with 3N hydrogen chloride in *n*-butanol was performed at 75 °C (rather than the normal 65 °C), for 15 min, to ensure complete esterification of the larger dicarboxylic acylcarnitines. The mixture was analysed by ESI-MS/MS with a precursor ion (85 Da) scan. Fig. 4 graphically illustrates the range of dicarboxylic acylcarnitines synthesized. The increase in ion intensity with size of the dicarboxylic acylcarnitine reflects the greater proportion of product ion with 85 Da relative to total product ions (cf. Figs. 2 and 3).

4. Conclusions

Dicarboxylic acylcarnitines (chloride salts) can be prepared with a purity of 85–95% by a condensation reaction between carnitine chloride and cyclic anhydrides or isopropylidene esters of dicarboxylic acids. These cyclic compounds are less reactive than acid chlorides and higher reaction temperatures are necessary. Consequently there is a practical limitation to the preparation of dicarboxylic acylcarnitines containing a dicarboxylic acid with more than 16 carbon atoms. Reaction temperatures above 75 °C, especially when trifluoroacetic acid is used as solvent, result in thermolysis of the carnitine ester. The solubility of larger dicarboxylic acylcarnitines in *n*-butanol, allowing their separation from unreacted carnitine in water, compensates for their poorer conversion. In all cases the dicarboxylic acylcarnitines are obtained as viscous oils or foamy solids after exhaustive vacuum drying as observed with acetoacetylcarnitine (Bohmer and Bremer, 1968). They are hygroscopic and should be stored in cold (−20 °C), dry conditions to avoid solvolysis of the carnitine ester. Additionally, in methanol solution, the carboxylic acid groups are slowly methylated.

Stable isotope labeled analogs of dicarboxylic acylcarnitines, suitable for their quantification by ESI-MS/MS, can be prepared containing isotope labels in either the dicarboxylic acid or the carnitine. The use of a stable isotope labeled standard of an acylcarnitine of a branched dicarboxylic acid, such as C12*br*DC and C16*br*DC, results in a small (typically 10%) underestimation of the acylcarnitine of its isomeric straight chain carboxylic acid. This was concluded from an examination of the product ion spectra of the butyl esters of a number of isomeric acylcarnitines (both mono- and dicarboxylic). The product ion spectra of branched acylcarnitines show greater fragmentation to the product ion with 85 Da, relative to higher mass product ions, than straight chain acylcarnitines.

It is expected that these synthesized isotope labeled dicarboxylic acylcarnitines will provide improved accuracy and precision in the measurement of the important metabolites C3DC and C5DC for tandem MS screening programs. The larger dicarboxylic acylcarnitines will be used as qualitative and quantitative tools for the differentiation of fatty acid oxidation disorders.

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