CHEMOENZYMATIC SYNTHESIS OF CARBON-14 LABELLED ANTIOXIDANTS

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SUMMARY

The syntheses of [^{14}C] labelled antioxidants are described. We developed an efficient synthetic methodology to prepare a series of labelled amides with antioxidant activity, starting from [^{14}C] KCN and alkyl or aryl halides. By a combination of nucleophilic displacement of halides by [^{14}C] cyanide, mediated by ultrasound and subsequent mild and selective enzymatic hydrolysis of the resulting nitriles, labelled carboxylic acids were obtained. Labelled amines were prepared by reduction of the respective nitriles. Availability of [^{14}C] KCN, efficient introduction of the label by ultrasound mediated reaction and selective and mild hydrolysis by comercially available nitrilase (Rhodococcus sp.), makes possible a wide range of applications of this methodology in the synthesis of functionalized labelled compounds.

Key words: **[^{14}C] labelled antioxidants, ultrasound, nitrilase**

INTRODUCTION

Amides containing the 3,5-di-t-butyl-4-hydroxyphenyl moiety were found to stabilize LDL (Low Density Lipoprotein) and to inhibit lipoxygenase/cyclooxygenase *in vitro* [1]. These compounds are potential antiinflammatory drugs and might inhibit LDL oxidation *in vivo*, generally considered to be a critical step in the development of atherosclerosis [2, 3].

To follow the fate of these compounds *in vivo* and to facilitate the study of their pharmacokinetics and metabolism in animals, isotopically labelled compounds were synthesized. [^{14}C] labelled nitriles were prepared by ultrasound mediated nucleophilic displacement of halides by [^{14}C] potassium cyanide. The labelled nitriles may be reduced chemically or hydrolyzed enzymatically and thus provide the same radioactive precursors for different amides. Our approach therefore allows the introduction of labelled carbon at the carboxylic acid function as well as at the amine function of the amides, thus yielding a higher specific radioactivity and facilitating the detection of most of the possible metabolites.

Hydrolysis of nitriles to carboxylic acids can be accomplished by use of two enzymes, a hydratase and

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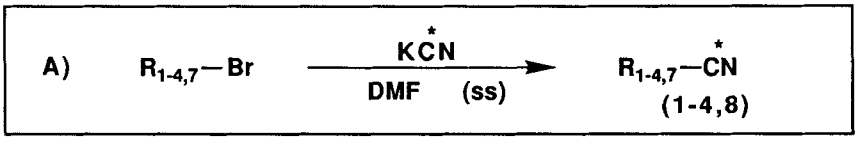
an amidase, (immobilized nitrilase complex Rhodococcus sp.(SP 409)) hydrolyzing nitriles sequentially *via* an amide intermediate [4-6].

EXPERIMENTAL
GENERAL

Chemicals were received from Aldrich (Steinheim/Germany) or Fluka (Buchs/Switzerland); [^{14}C] radiolabelled potassium cyanide (56.2 mCi/mmol) was from Amersham Buchler (Braunschweig/Germany); Nitrilase Rhodococcus sp. (SP 409) was obtained from Novo Nordisk (Bagsvaerd/Denmark). Liquid scintillation counting was performed with a Searle Isocap 300 liquid scintillation counter (Searle, nuclear department Chigago/USA) using Packard Insta-Gel liquid scintillation cocktail (Canberra Packard GmbH Frankfurt/Main/Germany). Radiochemical purity was checked by thin layer chromatography or by HPLC and was higher than 97% in all of the described products. Thin layer chromatography (TLC) plates RP-18 F_{254}s were purchased from E. Merck (Darmstadt/Germany); radiochromatograms were recorded using a Berthold LB 2723 TLC analyzer (Wildbad/Germany).

High performance liquid chromatography (HPLC) was performed using a gradient programmer GM 4000, a solvent delivery system constametric® 3000, a variable wavelength detector spectroMonitor®D and a calculator/printer CI-4100 (all LDC-Analytical, Gelnhausen/Germany). Analytical separations were performed using a RP 18 column from E. Merck (Darmstadt/Germany) (125x4 mm; Lichrospher® 100, 5µm). For preparative separations a 250x10 mm column (Spherisorb C18-5µm, Latek Heidelberg/Germany) equipped with a Beckman (München/Germany) Ultrasphere ODS 5µ (4.6 x 45 mm) precolumn was used. Proton magnetic resonance spectra were obtained with a WM-250 spectrometer (250 MHz ^{1}H, Bruker Physik AG Karlsruhe/Germany) using tetramethylsilane as an internal standard; chemical shifts are given on the δ scale. Mass spectra were obtained with a Mass spectrometer 311A (Varian Bremen/Germany). For sonification a Bransonic® Ultrasonic cleaner (Branson 2200, Branson/USA) was used; the bath temperature was kept at 40°C.

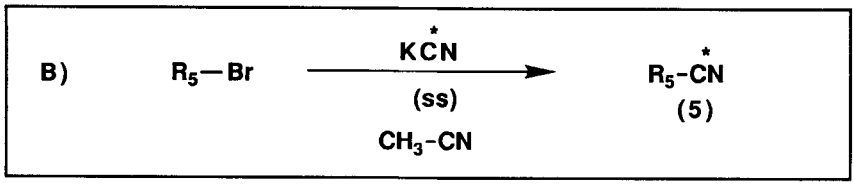
NUCLEOPHILIC DISPLACEMENT



Benzyl[^{14}C]cyanide (1)
Hydrocinnamo[^{14}C]nitrile (2),
3,5-Di-t-butyl-4-hydroxy-benzyl[^{14}C]cyanide (3),
Cyclohexan[^{14}C]carbonitrile (4)

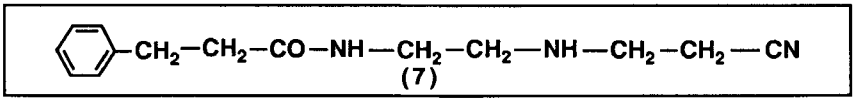
[^{14}C]-labelled potassium cyanide (0.15 mmol/56.2 mCi/mmol) was suspended in dimethylformamide (8 ml) and 0.31 mmol of the respective bromide (benzyl-bromide, phenylethyl-bromide, 2,6 di-t-butyl-benzyl-bromide, cyclohexyl-bromide) was added. The suspension was sonicated for one hour at

40°C, a three fold excess of potassium cyanide (unlabelled, 0.93 mmol) was added and the mixture was sonicated again for up to six hours at 40°C under argon. Insoluble salts were filtered off and the solvent evaporated. Thin layer chromatography and Rf values are listed in Table 1. Isolation and identification was performed using HPLC (linear gradient methanol : water (30:70) - methanol (100) or methanol : water (70:30) - methanol (100), 15 min, flow rate 1-3ml/min, columns see Experimental, General section). Chemical and radiochemical yields are listed in Table 2.



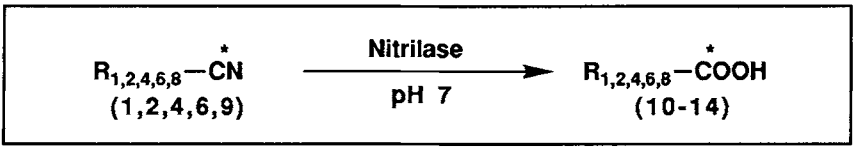
3-Aminopropio[^{14}C]nitrile (5)

[^{14}C]-labelled potassium cyanide (0.15 mmol/ 56 mCi/mmol) and 10,4 mg (0.16 mmol) potassium cyanide (unlabelled) was suspended in acetonitrile, 0.8 ml (0.31 mmol) of 2-bromoethylamine was added and the suspension sonicated for 5 min at 40°C. Insoluble salts were filtered off and the solvent evaporated. To facilitate the characterization of the 3-aminopropio[^{14}C]nitrile (**5**), the compound was dissolved in dry chloroform and 3-phenyl-propionylchloride was added to form the corresponding 3-phenyl-N-(2`-[^{14}C]cyanoethyl)-propionic-acid-3-amide (**6**). The nitrile (**7**) was isolated as a by product in 63% yield, when the sonification was extended to 60 min.



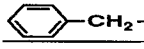
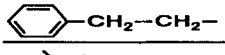
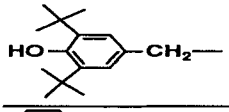
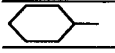
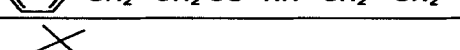
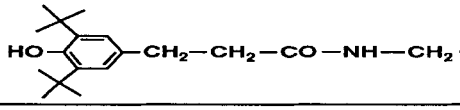
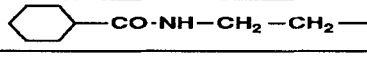
ENZYMATIC HYDROLYSIS OF [^{14}C]-NITRILES

(1, 2, 4, 6, 9)



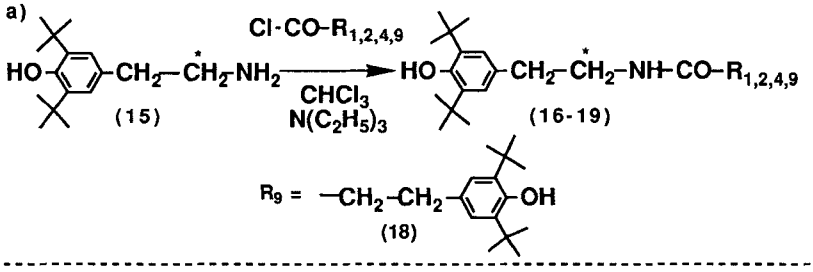
200 Mg nitrilase SP 409 (activity 391 HPU/g, 159 APU/g) was stirred as a suspension for one hour in phosphate buffer (10 ml, pH 7, room temperature) as described [5]. Then 4 mmol of the respective [^{14}C]-labelled nitrile (**1, 2, 4, 6, 9**) was added and the mixture was shaken for up to 7 days (200 U/min) at room temperature. The progress of the reaction was monitored by HPLC or TLC. When the reaction was completed, the solution was acidified with hydrochloric acid (pH 1) and extracted three times with 5 ml of chloroform. The organic layer was evaporated, the products (**10-14**) were isolated and quantified by high performance liquid chromatography (HPLC conditions as described above),

using authentic reference samples as external standards. The radiochemical yield was determinated by liquid scintillation counting (LSC) of the labelled compounds.

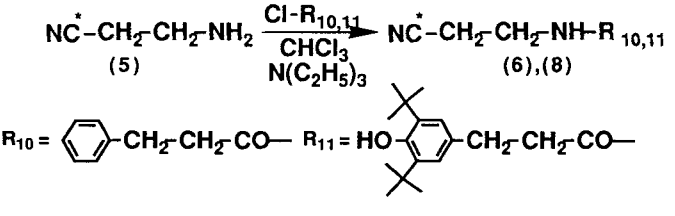
		R-$\overset{*}{C}$N	R-COOH
R_1		(1)	(10)
R_2		(2)	(11)
R_3		(3)	-
R_4		(4)	(12)
R_5	$NH_2-CH_2-CH_2-$	(5)	-
R_6		(6)	(13)
R_7		(8)	-
R_8		(9)	(14)

[^{14}C]-Amides (6, 8, 16, 17, 18, 19)

a)

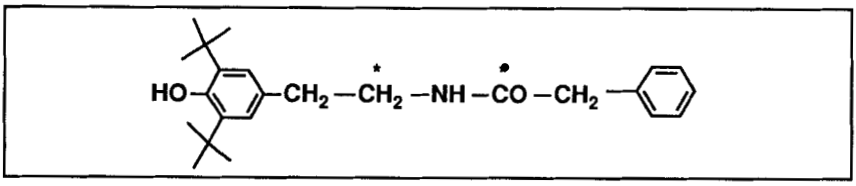


b)



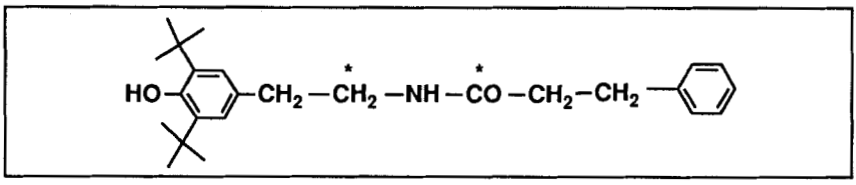
The amine [aminopropio[^{14}C]nitrile (**5**), 28 mg (0.4 mmol) or 2-(3,5-di-t-butyl-4-hydroxyphenyl)-[2-^{14}C]ethylamine (**15**), 100 mg (0.4 mmol)] were dissolved in dry chloroform and 1ml of triethylamine was added. The solutions were stirred at 0°C and the acid chlorides [(phenylacetyl-chloride, 3-phenyl-[1-^{14}C]propanoyl-chloride, cyclohexanoyl-chloride, 3-(3,5-di-t-butyl-4-hydroxyphenyl)-propanoyl-chloride, (3-phenyl-propionyl)-propionyl-chloride-3-amide, or (cyclohexanoyl)-propionyl-chloride-amide) (0.4 mmol)], dissolved in chloroform were added dropwise giving the respective [^{14}C]-labelled amides **6, 8, 16, 17, 18** and **19** after 3 h at room temperature. The products were analyzed using TLC system 1 (Rf-values see Table 1, yields see Table 4) and worked up as follows: The organic layers were extracted with hydrochloric acid / water (pH 1) and with NaOH/$NaHCO_3$ alkaline water (pH 10) and then analyzed using TLC system 2 (Rf values see Table 1). The chloroform layer was dried over magnesium sulfate and concentrated. The crude products (**16-19**) were dissolved in methanol and purified by HPLC as described above (flow rate 2 ml/min, HPLC retention times see Table 3).

N-[2-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[2-^{14}C]ethyl]-2-phenyl-[1-^{14}C]acetamide, (16):



^{1}H-NMR ($CDCl_3$) δ 7.27 (m, 3H); 7.17 (m, 2H); 6.89 (s, 2H); 5.51 (s, 1H); 5.10 (s, 1H); 3.53 (s, 2H); 3.43 (q, 2H); 2.66 (t, 2H); 1.40 (s, 18H).
MS (EI) m/e (%) 367 (3.54); 233 (17.93); 232 (100); 217 (10.29); 91 (16.80); 57 (19.50).

N-[2-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[2-^{14}C]ethyl]-3-phenyl-[1-^{14}C]propanoic-acidamide, (17):



^{1}H-NMR ($CDCl_3$) δ 7.27 (d, 3H); 7.19 (t, 3H); 6.94 (s, 2H); 5.40 (s, 1H); 5.10 (s, 1H); 3.45 (t, 2H); 2.96 (t, 2H); 2.66 (t, 2H); 2.44 (t, 2H); 1.42 (s, 18H).
MS (EI) m/e (%) 381 (5.52), 233 (17.84), 232 (100), 217 (28.4), 105 (10.2), 91 (15.8), 57 (21.3).

N-[3-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[1-14C] propanoic acid]-2-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[2-14C] ethylamide, (18);

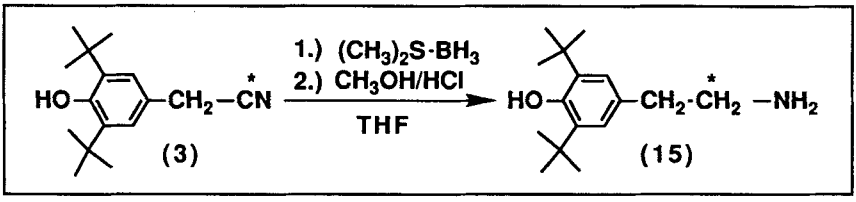
3,5-Di-t-butyl-4-hydroxy-benzylbromide

2.06 g (10 mmol) of 2,6-di-t-butyl-phenol and 1 equivalent of trioxane were dissolved in glacial acetic acid; 20 ml of hydrobromic acid (48 % in water) was added and the mixture stirred for three hours at 60°C. The product (3,5-di-t-butyl-4-hydroxy-benzyl-bromide) was separated from the aqueous phase, crystallized and used without further purification (yield: 2.03 g, 68%).

3,5-Di-t-butyl-4-hydroxy-benzyl[^{14}C]cyanide (3)

The benzyl[^{14}C]cyanide (3) was generated as described above, using 406.4 mg (1.6 mmol) of 3,5-di-t-butyl-4-hydroxybenzyl-bromide. Salts were removed by filtration and the filtrate, containing 3,5-di-t-butyl-4-hydroxy-benzyl[^{14}C]cyanide (3), was evaporated in *vacuo.* The products were analyzed using TLC system 1 (thin layer chromatography Rf-values see Table 1, chemical and radiochemical yields see Table 2).

2-(3,5-Di-t-butyl-4-hydroxy-phenyl)-[2-^{14}C]ethylamine (15)

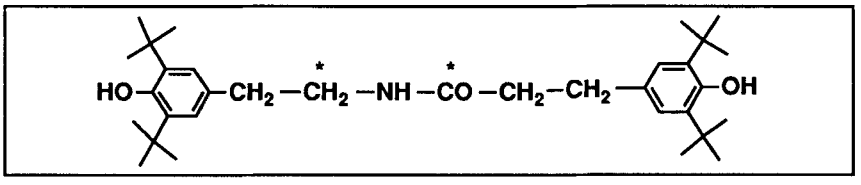


The [^{14}C]-labelled nitrile (3), 288 mg (1.17 mmol), was dissolved in tetrahydrofuran and 0.3 ml (3 mmol) borane-dimethyl-sulfide solution (0.01 mol/ml) in tetrahydrofuran was added. The solution was refluxed under argon for 2.5 hrs and the reaction monitored by thin layer chromatography. (a 20 µl sample was taken for thin layer chromatography, hydrolyzed with 1 N hydrochloric acid, made alkaline (pH 9) and extracted with chloroform, using TLC system 1 (Rf-values see Table 1)).

When the reaction was completed, 15 ml of a methanol/hydrochloric acid mixture (3:1) was added and the mixture refluxed for another hour. The methanol was evaporated under reduced pressure and the evaporation was repeated with three portions of 20 ml methanol.

The residue was dissolved in buffer ($NaOH/NaHCO_3$, pH 9) and the [^{14}C]-labelled amine (15) was extracted twice with 20 ml of diethylether. The aqueous phase then was extracted continously two times for one hour with 100 ml of diethylether. The ether fractions were combined, dried over magnesium sulfate and the solvent removed (yield: 216 mg, 74 %).

N-[3-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[1-^{14}C] propanoic-acid]-2-(3,5-Bis-(1,1-dimethylethyl)-4-hydroxyphenyl)-[2-^{14}C] ethylamide, (18);
The reaction conditions were as described under "amides". Rf-values see Table 1 and yields see Table 2 and 4.



^{1}H-NMR ($CDCl_3$) δ 7.00 (s,2H); 6.97 (s, 2H); 5.44 (s, 1H); 5.10 (s, 1H); 5.07 (s, 1H); 3.47 (t, 2H); 2.87 (t, 2H); 2.70 (t, 2H); 2.42 (t, 2H); 1.42 (s, 36H).
MS (EI) m/e (%) 509 (6.10), 277 (23.41), 233 (19.34), 232 (100), 217 (17.69), 57 (23.90).

Table1: TLC retention times relative to solvent front

compounds	TLC 1	TLC 2	compounds	TLC 1	TLC 2
(1)	0.82	-.-	(9)	0.86	-.-
(2)	0.60	-.-	(15)	0.15	-.-
(3)	0.67	-.-	(16)	-.-	0.19
(4)	0.80	-.-	(17)	-.-	0.24
(5)	0.81	-.-	(18)	-.-	0.25
(6)	0.74	-.-	(19)	0.89	0.18
(8)	0.76	-.-			

TLC 1: RP 18 plates; methanol : water, 9 : 1

TLC 2: Polygram Sil G/UV 254; hexane : ethylacetate, 85 : 15

Table 2: Chemical and radiochemical yields of nitriles (1-6, 8, 9),carboxylic acids (10-14) and the amine (15)

compounds	YIELD (%)		compounds	YIELD (%)	
	C^1	R-C^2		C^1	R-C^2
(1)	76.1	95.9	(9)	87.2	99.2
(2)	73.2	97.6	(10)	48.4	51.9
(3)	71.0	89.9	(11)	40.1	39.8
(4)	23.2	60.1	(12)	51.3	45.7
(5)	55.7	96.7	(13)	45.6	44.5
(6)	78.2	98.5	(14)	39.4	35.8
(8)	82.9	99.1	(15)	81.5	79.5

C^1 : chemical; **R-C^2** : radiochemical

Table 3: HPLC retention times (min)

compounds	HPLC 1	compounds	HPLC 1
(16)	**12.47**	(18)	**14.69**
(17)	**12.95**	(19)	**12.32**

HPLC 1: RP 18 column (125x4 mm; Lichrosphere® 100; 5µm), E. Merck

Table 4: Chemical and radiochemical yields of amides (16-19)

compounds	YIELD (%)		compounds	YIELD (%)	
	C^1	R-C^2		C^1	R-C^2
(16)	**77.1**	**76.4**	(18)	**72.3**	**68.6**
(17)	**71.5**	**68.5**	(19)	**73.8**	**71.3**

C^1 : chemical; **R-C^2** : radiochemical

RESULTS AND DISCUSSION

The synthesis of labelled antioxidants started with the displacement of bromine in benzyl or alkyl halides by [^{14}C] cyanide. Incorporation of labelled carbon by means of [^{14}C] potassium cyanide usually requires high reaction temperatures and long reaction times (except for benzylic halides) and sometimes even fails completely. Reaction of chlorocyclohexane with sodium cyanide, for example, does not yield any of the desired nitrile [7]. Reactions with aluminia or silica gel supported reagents [8] usually require a large excess of reagent.

The use of ultrasound, however, allows the preparation of labelled nitriles in good yields at mild temperatures (see Table 2). Moreover, the displacement of bromine by [^{14}C] cyanide (compounds **1-9**) can be performed in short to moderate reaction times (see Table 3). In a typical reaction potassium cyanide and the respective alkyl/benzyl bromides were combined in dimethylformamide and the suspension sonicated in a conventional ultrasound cleaning bath for 15 to 150 min. To optimize the radiochemical yield, [^{14}C] potassium cyanide was sonicated first with an excess of the respective bromide for up to 60 min. The reaction then was completed by sonification with an excess of unlabelled potassium cyanide. Thus benzyl-bromide, phenylethyl-bromide, (2,6-di-t-butyl-benzyl)-bromide and 2-bromoethylamine were readily transformed to the corresponding nitriles (**1-9**) in good to excellent yields (see Table 2).

Chemical hydrolysis of nitriles usually requires drastic basic or acidic conditions which are often incompatible with molecules carrying sensitive functionalities (for example compound (**8**)). Moreover, side reactions may occur and result in decreased yields: acidic hydrolysis of t-butylated phenols results in alkyl-migration and alkaline hydrolysis of the corresponding benzylnitriles affords very modest yield of the desired carboxylic acid (**13**). Both procedures are not suitable for sensitive amide-like compounds (**8**). Thus, enzymatic hydrolysis of nitriles under mild conditions (room temperature and pH 7) is an attractive alternative to the conventional chemical procedure. The immobilized enzyme system prepared from Rhodococcus sp. is commercially available, contains nitrilase-hydratase and amidase activity and has been demonstrated to transform a range of substrates [6]. For the potential hydrolysis of all nitrile substrates (**1-9**) to carboxylic acids, the compounds were dissolved (or suspended) in phosphate buffer (0.1 μM, pH 7, 10-30 ml) giving a final concentration of 200 mM. Optimal reaction times were determined by TLC and HPLC analysis and products (**10-14**) were isolated by chloroform or ether extraction of the acidified solution (or suspension). Nitriles bearing the 2,6-di-t-butyl moiety (**3/8**), were not hydrolyzed, probably due to steric hindrance of the bulky t-butyl groups. Labelled or non labelled acids (**10-14**) were transformed to acid chlorides by reaction with phosphorus pentachloride and the labelled or non labelled amines (**5/15**) were added in the presence of triethylamine to give the respective amides (**6/7, 16-19**). The [^{14}C] labelled amine (**15**) was obtained by reduction of the nitriles with borane dimethylsulfide in tetrahydrofuran according to a published procedure [9]. In the amides (**16-19**) both carbons adjacent to the N-atom are labelled. These compounds will retain the label at both breakdown products after a possible amide hydrolysis by proteases *in vivo*.

In conclusion, a combination of ultrasonification for the nucleophilic displacement of ethyl bromides with CN^- and enzymatic hydrolysis of the nitriles appears to be of general value for the synthesis of [^{14}C] labelled carboxylic acid derivatives, especially for the preparation of highly functionalized compounds.

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