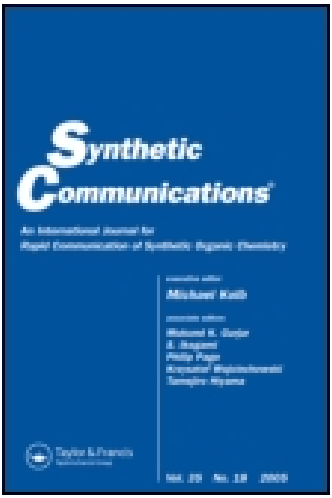

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Improved Yields in the Synthesis of Spin-Labeled Fatty Acids

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ABSTRACT

Paramagnetic amide side products (**6a–g**) have been isolated from the reaction mixture in the synthesis of spin-labeled fatty acids of the doxyl type. After their hydrolysis to the corresponding acid, **7**, the overall yield of spin-labeled fatty acids is significantly increased compared with published procedures.

Key Words: Amide hydrolysis; Fatty acid spin probe.

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INTRODUCTION

Fatty acid methyl esters and, especially, the corresponding acids containing the *N*-oxyl-4,4-dimethyl-oxazolidine (doxyl) group in the alkyl chain, are useful molecular tools in electron spin resonance (ESR) spectroscopy as lipophilic and amphiphilic spin probes for studying the structure and dynamics of complex systems, especially phospholipid bilayers and biological membranes.[1–4] The interaction of fatty acid spin labels with membrane components reflects the structural and dynamic properties of the local environment, accounting for their wide use as spin probes. They are also used for preparing spin-labeled phospholipids[4–7] and biologically interesting amphiphilic molecules.[8]

The doxyl group is usually prepared by oxidation of an oxazolidine compound (**2**, Fig. 1) using 3-chloroperoxybenzoic acid.[4,9] The **2** is obtained by condensation of a fatty acid ketoester (**1**) with 2-amino-2-methylpropanol. After isolation and purification, doxyl fatty acid ester (**5**) is hydrolyzed under standard conditions[1,5] to spin-labeled acid (**7**).[1,5,6] The condensation of **1** with amino alcohol is normally carried out in boiling benzene, toluene, or xylene in the presence of para-toluenesulfonic acid as a catalyst (Fig. 1).[1,4,5] Although the water is continuously removed by a Dean–Stark water separator and a large excess of amino alcohol is usually used, the formation of **2** is rather slow, and after 7–9 days of heating (reflux), considerable amounts of the starting **1** are still present. According to the published procedures,[1,5] **5** is isolated

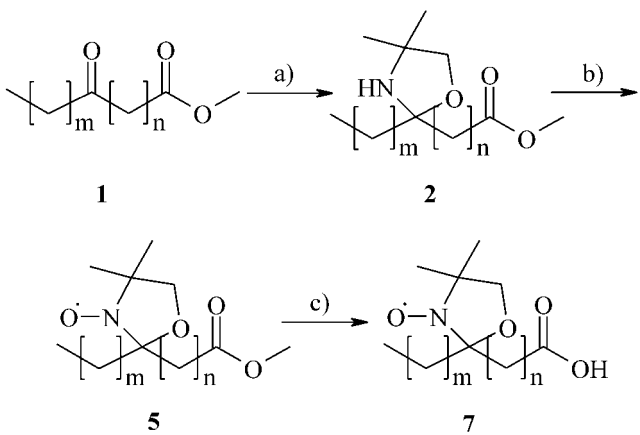

Figure 1. a) 2-amino-2-methylpropanol, $TsOH \cdot H_2O$, toluene, reflux, and water separation by Dean–Stark separator; b) 3-chloroperoxybenzoic acid, ether; c) 1 M NaOH, dioxane.

in 15–30% overall yield. Ester (**5**) is then practically quantitatively hydrolyzed to acid **7**. Thus, any increase in the overall yield of preparation of **7** will be acceptable, especially if **7** is used as a starting material for the synthesis of more complex spin probes.

RESULTS AND DISCUSSION

During the synthesis of many different spin-labeled fatty acids (**7**), we observed that after oxidation there are at least two paramagnetically active compounds present in considerable amounts in the reaction mixture. The fast-moving compound eluted with petroleum ether–ether (3 : 1 v/v) from a silica gel column (**5**) is already known,[1,5] while a second paramagnetically active compound, under the stated conditions, stays bound to the silica gel. Because of the low overall yield of doxyl acids (**7**) and because only the isolation of doxyl esters (**5**) is described in published papers, we have studied the reaction mixture resulting from the oxidation step more carefully. We isolated and characterized two additional compounds from the silica gel column. We found that, under the published conditions,[1,5] oxoester (**1**) reacts with amino alcohol to give (Fig. 2) oxazolidine esters **2a–g**, oxoamides **3a–g** and oxazolidine amides **4a–g**.

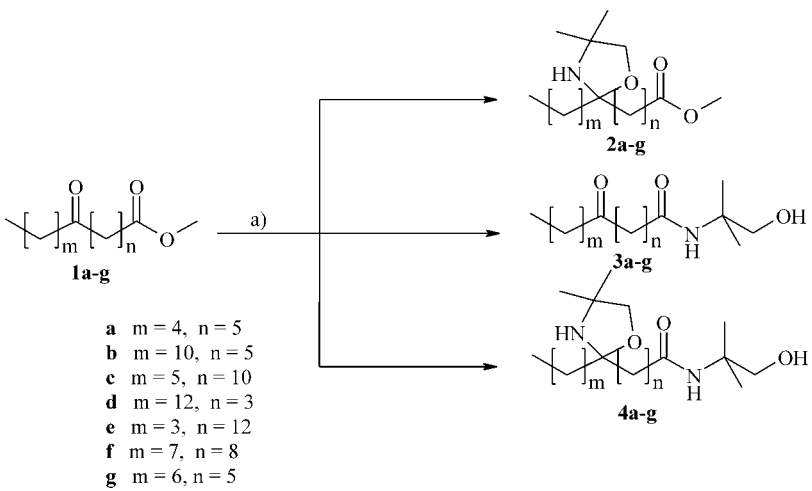

Figure 2. a) 2-amino-2-methylpropanol, $TsOH \cdot H_2O$, toluene, reflux, and water separation by Dean–Stark separator.

Because the isolation and purification of **2** from the mixture is complicated, the published procedures use the mixture directly in the oxidation step. After separation on the silica gel column, **5** is isolated with 15–30% overall yield, while other side products, **6** and **3**, have been ignored (Fig. 3).[1,5] To improve the relatively low yield of **7**, we purified and characterized the other two side products that are formed in the condensation step in considerable amounts. After elution of **7**, the yellow residue at the top of the column was eluted with a more polar eluant, ether. The corresponding yellow band was collected and found to be composed of two compounds, one yellow and paramagnetic **6a–g**, and the other nonparamagnetic oxoamide **3a–g**. Both compounds have similar retention factors. On thin-layer chromatography (TLC), **6** is readily seen under ultraviolet (UV), while **3** is visualized by spraying with a reagent for ketones, 2,4-dinitrophenylhydrazine. The mixture of **3** and **6** was further resolved by size exclusion chromatography (Sephadex LH-20, ethanol as eluant). The analytically pure doxyl amide **6a–g** was isolated as an orange-yellow viscous oil or solid, in yields listed in Table 1. To prove the structure of **6a–g**, a sample of amide (**6d**) was hydrolyzed (Fig. 3). The isolated spin-labeled fatty acid (**7**) was shown to be identical to a sample synthesized from **5** according to Ref.[6].

EXPERIMENTAL

General

Chemicals were obtained (Acros, Aldrich, Fluka, Merck, Jannsen, and Sigma) and used without further purification. Solvents were used without

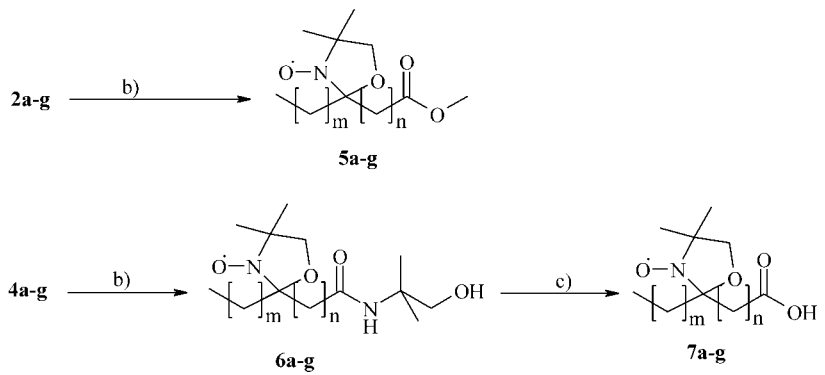

Figure 3. b) 3-chloroperoxybenzoic acid, ether; c) 1 M NaOH, dioxane, 60°C.

Table 1. Yields of spin-labeled compounds.

Compound (m,n)	Yield[a] of **6**[b] (%)	Yield[a] of **5** (%)	Theoretical overall yield of **7** (%)
a(4,5)	25.4	28.1	53
b(10,5)	22.5	28.5	51
c(5,10)	18.3	30.2	48
d(12,3)	20.0	17.2	37
e(3,12)	24.2	20.8	45
f(7,8)	16.3	20.4	37
g(6,5)	23.8	25.7	49

[a]Of analytically pure material.
[b]All compounds **6a–g** have a_N (ethanol) = 1.485 mT.

additional purification or drying. Ketoesters (**1a–g**) were prepared according to known procedures.[1,5] Analytical TLC plates (Merck, silica gel 60 F_{254}) were used to monitor the reactions. Silica gel 60 (70–230 mesh, Merck) was used for column chromatography. Size exclusion chromatography was carried out on Sephadex LH-20 (Pharmacia). Mass spectra were obtained with a VG-Analytical Autospec Q mass spectrometer with EI ionization (MS Centre, Jožef Stefan Institute). Infrared (IR) spectra were recorded on a Perkin–Elmer FT-IR 1600 spectrometer. Electron paramagnetic resonance (EPR) spectra of nitroxide solutions were measured on a BRUKER X-band continuous wave ESR (CW-ESR) spectrometer ESP 300 (EPR Centre, Jožef Stefan Institute) at room temperature in a glass capillary (1 mm inner diameter) and at 10 mW microwave power.

General Procedure (Modified from Refs. [1] and [5])

To 250 mL of toluene were added methylketoester **1a–g** (0.05 mol), 50 mL (0.5 mol) of 2-amino-2-methylpropanol, and 50 mg (0.26 mmol) of *p*-toluenesulfonic acid monohydrate. The mixture was heated at reflux for 9 days using a Dean–Stark trap for water removal. The toluene phase was then washed (4 × 150 mL) with saturated sodium bicarbonate solution and with brine (2 × 150 mL), then dried (Na_2SO_4). After evaporating the toluene under reduced pressure, the colorless, viscous liquid containing compounds **1a–g**, **2a–g**, **3a–g**, and **4a–g** was dissolved in 300 mL of ether and cooled in an ice bath. 50 mL ether containing 0.055 mol of 3-chloroperoxybenzoic acid (80–90%, Jannsen) was added over a period of 2 h. The mixture was allowed to stand for 12 h, after which the ether phase was

washed (8 × 150 mL) with saturated sodium bicarbonate, (2 × 100 mL) with water, and with brine (2 × 100 mL), then dried (Na_2SO_4). After evaporating the ether under reduced pressure, the yellow, viscous oil was chromatographed on 500 g of silica gel, using ether/petrolether = 1/3 (v/v) as eluent. The starting compound **1a–g** was eluted before the yellow colored zone. The center portion of the first fast-moving yellow band was collected, yielding 0.0075 to 0.0150 mol of **5a–g** as an orange oil. The yellow residue at the top of the column was eluted with ether, and the main portion of the second moving yellow band was collected. The mixture of compounds **3a–g** and **6a–g** was further separated by size exclusion chromatography on Sephadex LH-20 using ethanol (96%) as eluent, yielding 0.0075 to 0.0125 mol of analytically pure **6** as an orange oil (**6a**, **6b**, **6c**, **6e**, **6f**, and **6g**) or yellow solid (**6d**).

N-(2-hydroxy-1,1-dimethylethyl)-6-(3-oxyl-4,4-dimethyl-2-penthyl-1, 3-oxazolidin-2-yl)hexanamide (**6a**)

IR (NaCl): $\nu = 3310, 2932, 2870, 1650, 1548, 1462, 1365, 1263, 1184, 1055\,cm^{-1}$; MS-EI m/e (relative intensity): 372 ($(M + 1)^+$, 4), 353 (5), 340 (14), 301 (6), 286 (24), 228 (8), 212 (12), 186 (30), 151 (10), 129 (11), 95 (10), 90 (20), 72 (15), 58 (100); high-resolution mass spectra (HRMS) calculated for $C_{20}H_{40}N_2O_4$ $(M + 1)^+$ 372.2988, found 372.2998.

N-(2-hydroxy-1,1-dimethylethyl)-6-(3-oxyl-4,4-dimethyl-2-undecyl-1, 3-oxazolidin-2-yl)hexanamide (**6b**)

IR (NaCl): $\nu = 3307, 2926, 2854, 1650, 1548, 1462, 1366, 1263, 1184, 1056\,cm^{-1}$; MS-EI m/e (relative intensity): 456 ($(M + 1)^+$, 14), 437 (6), 424 (11), 370 (33), 301 (15), 283 (6), 270 (30), 229 (10), 212 (10), 129 (10), 90 (23), 81 (6), 72 (15), 58 (100); HRMS calculated for $C_{26}H_{52}N_2O_4$ $(M + 1)^+$ 456.3927, found 456.3905.

N-(2-hydroxy-1,1-dimethylethyl)-11-(2-hexyl-3-oxyl-4,4-dimethyl-1, 3-oxazolidin-2-yl)undecanamide (**6c**)

IR (NaCl): $\nu = 3309, 2927, 2855, 1649, 1546, 1461, 1364, 1266, 1057\,cm^{-1}$; MS-EI m/e (relative intensity): 456 ($(M + 1)^+$, 2), 437 (1), 424 (10), 370 (30), 352 (11), 339 (11), 282 (17), 242 (10), 200 (37), 129 (16), 113 (14), 90 (26), 72 (19), 58 (100); HRMS calculated for $C_{26}H_{51}N_2O_4$ $(M)^+$ 455.3849, found 455.3862.

N-(2-hydroxy-1,1-dimethylethyl)-4-(3-oxyl-4,4-dimethyl-2-tridecyl-1, 3-oxazolidin-2-yl)butanamide (**6d**)

IR (NaCl): $\nu = 3297$, 2919, 2851, 1628, 1550, 1466, 1380, 1192, 1057 cm^{-1}; MS-EI m/e (relative intensity): 456 ($(M + 1)^+$, 2), 438 (4), 424 (8), 370 (37), 352 (6), 298 (39), 281 (34), 273 (20), 253 (25), 239 (7), 214 (8), 201 (24), 184 (59), 126 (30), 113 (34), 97 (25), 90 (19), 83 (27), 69 (47), 58 (100); HRMS calculated for $C_{26}H_{52}N_2O_4$ $(M + 1)^+$ 456.3927, found 456.3945.

N-(2-hydroxy-1,1-dimethylethyl)-13-(2-butyl-3-oxyl-4,4-dimethyl-1, 3-oxazolidin-2-yl)tridecanamide (**6e**)

IR (NaCl): $\nu = 3319$, 2926, 2854, 1650, 1548, 1462, 1364, 1263, 1056 cm^{-1}; MS-EI m/e (relative intensity): 456 ($(M + 1)^+$, 4), 440 (2), 425 (7), 399 (11), 370 (20), 352 (8), 310 (15), 270 (8), 228 (6), 172 (47), 156 (10), 129 (14), 113 (11), 97 (7), 90 (22), 72 (20), 58 (100); HRMS calculated for $C_{26}H_{52}N_2O_4$ $(M + 1)^+$ 456.3927, found 456.3945.

N-(2-hydroxy-1,1-dimethylethyl)-9-(3-oxyl-4,4-dimethyl-2-octyl-1, 3-oxazolidin-2-yl)nonanamide (**6f**)

IR (NaCl): $\nu = 3308$, 2927, 2856, 1645, 1543, 1463, 1364, 1263, 1057 cm^{-1}; MS-EI m/e (relative intensity): 456 ($(M + 1)^+$, 3), 437 (1), 424 (16), 370 (45), 352 (11), 343 (20), 311 (15), 281 (10), 271 (13), 254 (20), 228 (55), 214 (16), 142 (14), 129 (23), 90 (38),72 (27), 58 (100); HRMS calculated for $C_{26}H_{51}N_2O_4$ $(M)^+$ 455.3849, found 455.3851.

N-(2-hydroxy-1,1-dimethylethyl)-6-(2-heptyl-3-oxyl-4,4-dimethyl-1, 3-oxazolidin-2-yl)hexanamide (**6g**)

IR (NaCl): $\nu = 3311$, 2930, 2858, 1650, 1562, 1462, 1365, 1263, 1055 cm^{-1}; MS-EI m/e (relative intensity): 400 ($(M + 1)^+$, 21), 381 (19), 368 (45), 314 (73), 301 (33), 283 (24), 269 (16), 242 (11), 229 (27), 214 (76), 198 (15), 179 (14), 142 (17), 129 (29), 107 (17), 90 (47), 81 (18), 72 (31), 58 (100); HRMS calculated for $C_{22}H_{44}N_2O_4$ $(M + 1)^+$ 400.3301, found 400.3310.

General Procedure for the Hydrolysis of Amides 6a–g

0.1 g of amide **6a–g** was dissolved in 1.5 mL of dioxane, and 1 mL of 1 M NaOH was added. The reaction mixture was stirred for 24 h at 60°C. The chilled solution was brought to pH 1 with 1 M HCl, 10 mL of water was added, and the solution was extracted with ethyl acetate. The ethyl acetate phase was dried (Na_2SO_4), and the solvent was removed under reduced pressure to yield the corresponding spin-labeled fatty acid **7a–g** in almost quantitative yield.

4-(3-Oxyl-4,4-dimethyl-2-tridecyl-1,3-oxazolidin-2-yl) butanoic acid (**7d**)

IR (NaCl): $\nu = 3496, 3298, 2919, 2850, 1713, 1470, 1429, 1216, 1142, 1044, 944, 782\,cm^{-1}$; MS-EI m/e (relative intensity): 384 ($(M)^+$, 3), 368 (2), 338 (3), 328 (12), 298 (46), 281 (100), 253 (51), 211 (11), 202 (72), 186 (16), 142 (21), 130 (50), 112 (32), 97 (22), 87 (45), 71 (25), 56 (63); HRMS calculated for $C_{22}H_{42}NO_4$ $(M)^+$ 384.3114, found 384.3125.

6-(2-Heptyl-3-oxyl-4,4-dimethyl-1,3-oxazolidin-2-yl) hexanoic acid (**7g**)

IR (NaCl): $\nu = 2930, 2858, 1711, 1464, 1261, 1052\,cm^{-1}$; MS-EI m/e (relative intensity): 328 ($(M)^+$, 10), 312 (2), 282 (4), 272 (36), 243 (19), 230 (61), 225 (93) 214 (69), 207 (30), 198 (14), 179 (44), 158 (39), 142 (51), 129 (88), 109 (40), 97 (46), 86 (25), 81 (31), 69 (55), 56 (100); HRMS calculated for $C_{18}H_{34}NO_4$ $(M)^+$ 328.2488, found 328.2490.

N-(2-hydroxy-1,1-dimethylethyl)-10-oxooctadecanamide (**3f**) as a representative compound of oxoamides **3a–g**

IR (NaCl): $\nu = 3165, 2968, 2718, 2027\ 1627, 1552, 1466, 1365, 1098\,cm^{-1}$; MS-EI m/e (relative intensity): 370 ($(M+1)^+$, 11), 352 (3), 338 (31), 327 (6), 298 (8), 281 (9), 256 (7), 228 (24), 214 (29), 184 (6), 144 (12), 131 (21), 113 (6), 97 (7), 90 (12), 83 (6), 72 (17), 58 (100); HRMS calculated for $C_{22}H_{44}NO_3$ $(M+1)^+$ 370.3321, found 370.3330.

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