Crosslinking 2,5-Dihydro-1*H*-pyrrol-1-yloxyl Spin-Label Reagents Bifunctionalized with Azidophthalimidyl and Methylthiosulfonyloxy Groups

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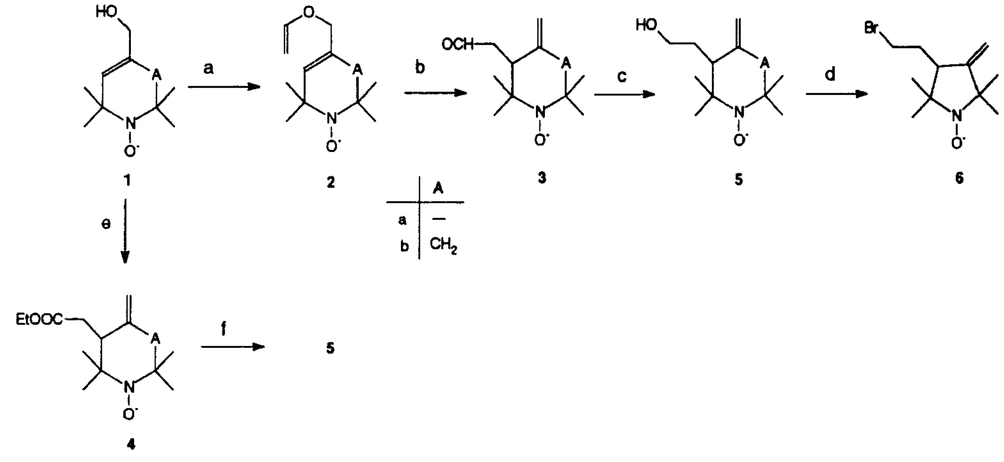
Oxy-Cope and ortho ester Claisen rearrangements of 5- and 6-membered *N*-oxyl radicals were carried out. The resulting more stable 5-membered bromo derivative **6** was used in the synthesis of bifunctionalized crosslinking photo- and thiol-label **12**.

Spin labels are often employed in motional studies of macromolecules and, particularly, biomolecules such as nucleic acids and proteins.[1,2] Both the overall mobility as well as segmental motions are of interest. The label is generally attached via its functionalization to reactive residues in the macromolecule, e.g., thiol groups of cysteine residues in proteins. Such studies are limited, however, owing to residual motion of the label with respect to the macromolecule. Relatively fast rotation around the linking bond creates a major problem. In the present study, we describe the synthesis of 3,4-hetero-bifunctionalized 2,5-dihydro-1*H*-pyrrol-1-yloxyl radicals as bifunctional spin labels[3] which would eliminate this problem. Attachment to thiols could be via the methanethiosulfonate group, establishing a cleavable mixed disulfide bond,[4-6] followed by non-specific reaction of nitrene with a second site. The nitrene can be generated photochemically from the azide function as reported earlier.[7,8]

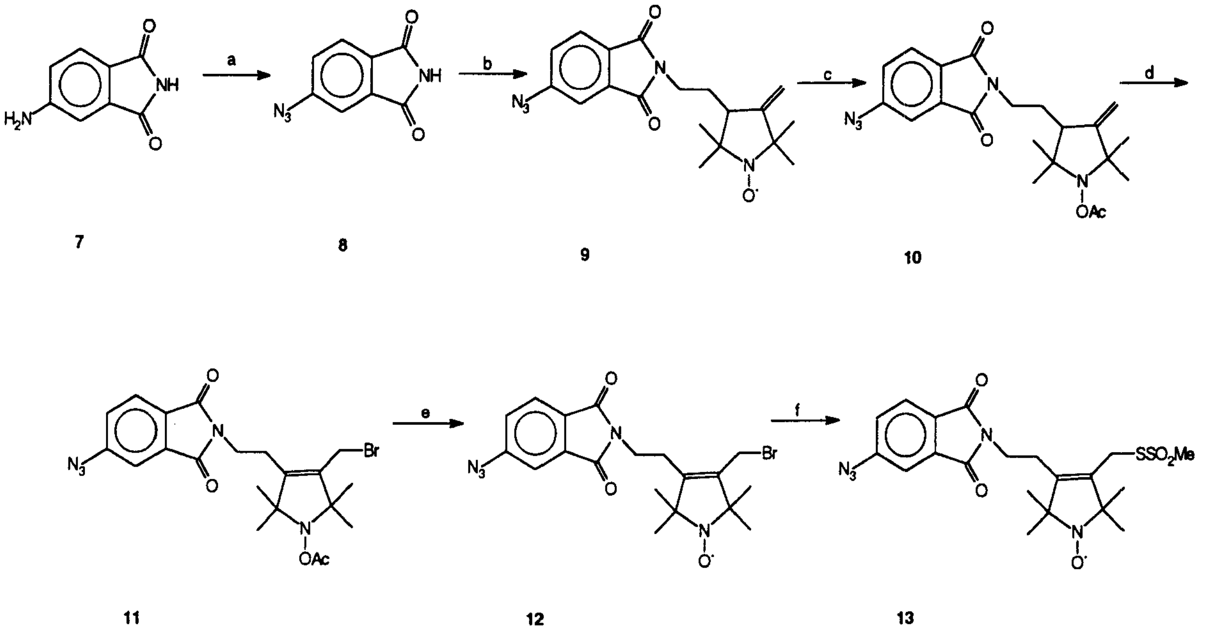
The exo olefinic aldehydes **3a,b** can be formed by oxy-Cope rearrangement[9,10] of allyl vinyl ether **2a,b**, prepared from allylic alcohols **1a,b** and ethyl vinyl ether. The synthesis of compound **4a**, reported in our recent paper, was achieved via an ortho ester Claisen rearrangement.[11]

The reaction is now extended to the 6-membered-ring compound **4b**. The reduction of esters **4a,b** and aldehydes **3a,b** with $NaBH_4$ gave alcohols **5a,b**.[12] Although both **5a** and **5b** are potentially functionalizable, because of the significantly higher stability of 5-membered-ring nitroxide free radicals,[13,14] the following reactions were applied only to the 5-membered nitroxide radicals (Scheme 1). Alcohol **5a** was converted into bromide **6** via the mesylate, followed by coupling of **6** with 4-azidophthalimide **8**, prepared from 4-aminophthalimide[15] **7**, yielding compound **9** functionalized with a photoactivatable arm. Although the application of photoactivatable spin labels has been studied,[7] the azidophthalimidyl function has not yet been reported as a photoactivatable arm. To introduce the allylic bromide function, compound **8** was reduced selectively to its *N*-hydroxyl derivative with ascorbic acid, and the labile hydroxyl amine was then acetylated to the diamagnetic *O*-acetyl compound **10** to avoid its spontaneous re-oxidation to the more stable 1-yloxyl radical, since the allylic bromination is applicable only in the absence of radicals. The allylic bromination of the bridgehead olefin bond is achievable either with *N*-bromosuccinimide (NBS) or 1,3-dibromo-5,5-dimethylhydantoin (DDH), giving compound **11** via allylic rearrangement, as recently observed.[11] The *N*-acetoxy compound **11** was deprotected with Zemplen's method[16,17] (1 equiv MeOH in THF with a catalytic amount of sodium) yielding paramagnetic bifunctionalized compound **12**, which was converted further with sodium methanethiosulfonate to the hetero-bifunctionalized nitroxide free radical reagent **13** with a photoactivatable and a cleavable SH-specific arm capable of spin labeling of two different functional groups in proteins in a sequential way (Scheme 2).



(a) CH_2=CHOEt / $Hg(OAc)_2$ / 33 °C / 16 h / 51-58 %. (b) xylene / 144 °C / 20 h / 27-32 %. (c) $NaBH_4$ / EtOH / 40 °C / 30 min / 80-85 %.
(d) 1. MsCl / Et_3N / CH_2Cl_2 / 0 °C → r.t. / 1h, 2. LiBr / $(H_3C)_2CO$ / 56 °C / 1h / 90 %. (e) $CH_3C(OEt)_3$ / EtCOOH / 140 °C / 8 h / 60-72 %.
(f) $NaBH_4$ / *t*-BuOH / MeOH / 83 °C / 3-4 h / 42-65 %.

Scheme 1



(a) 1. $NaNO_2$ / conc. HCl / 30 min / 0 °C, 2. NaN_3 / 30 min. / 0 °C. (b) 6 / K_2CO_3 / KOH / 18-crown-6 / dioxane / 100 °C / 18 h / 35 %. (c) 1. ascorbic acid / dioxane / H_2O / 40 °C / 15 min, 2. AcCl / Et_3N / $CHCl_3$ / 0°C → r. t. / 1 h / 81 %. (d) NBS / benzoyl peroxide / CCl_4 / 76 °C / 14 h / 56 %. (e) NaOMe / THF / 25 °C /30 min / 28 %. (f) $NaSSO_2Me$ / dioxane / H_2O / 100 °C / 30 min / 66 %.

Scheme 2

In conclusion, the synthetic route described herein enables us to obtain bifunctional semicleavable reagents that can be used for spin-labeling studies on biomolecules.

Melting points were determined on a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N) were performed on Heraeus Micro U/E apparatus or (Hal) were carried out titrimetrically by Schöniger's method. The IR (Specord 75) spectra of the compounds were in each case consistent with the assigned structures. ^1H NMR spectra were recorded with a Bruker AC-250 spectrometer at 250 MHz. Chemical shifts are given in ppm, relative to TMS internal standard, J values are given in Hz. Mass spectra were taken on a Finnigan MAT 8430 mass spectrometer/SS300 data acquisition system. Operating conditions: $U_{acc} = 3$ kV, $E_{el} = 70$ eV, $I_{el} = 0.5$ mA, T_{ion} source = 250 °C, $R = 1250$. Samples were introduced via the direct insertion probe. The evaporation temperatures of the samples were 50–250 °C, and were controlled within ± 1 °C. Assignments were corroborated by high-resolution mass measurements made at $R = 10000$ by the peak matching technique, with perfluorokerosene as the reference material.

Flash column chromatography on silica gel was performed with Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 x 20 x 0.2 cm) coated with Merck Kieselgel GF_{254}.

Compounds **1a**,[18] **1b**,[6] **4a**,[11] **7**[15] were prepared according to published procedures. The physical and spectral data of all new compounds are listed in the Table.

Allylic Vinyl Ethers 2a,b; General Procedure:

A mixture of allylic alcohol **1a,b** (20.0 mmol), ethyl vinyl ether (11.53 g, 160 mmol) and $Hg(OAc)_2$ (318 mg, 1 mmol) was refluxed for 16 h, then evaporated to dryness. The residue was dissolved in Et_2O (30 mL) washed with water (10 mL), and dried ($MgSO_4$). Evaporation of the filtrate gave crude allylic vinyl ether **2a,b**, which was purified by flash chromatography (silica gel; hexane–Et_2O).

3-Vinyloxymethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-yloxy Radical (**2a**):

Yield: 2.27 g (58 %); R_f 0.48 (hexane–Et_2O, 2:1) (TLC); oil.

4-Vinyloxymethyl-1,2,5,6-tetrahydro-2,2,6,6-tetramethylpyridin-1-yloxy Radical (**2b**):

Yield: 2.14 g (51 %); R_f 0.56 (hexane–Et_2O, 2:1) (TLC); oil.

ω-Alkenyl Aldehydes 3a,b; General Procedure:

A solution of allylic vinyl ether **2a,b** (10.0 mmol) was refluxed in xylene for 20 h, then solvent was evaporated in vacuo. The brown residue was purified by flash chromatography (hexane–Et_2O) to give aldehyde **3a,b**.

3-Formylmethyl-2,2,5,5-tetramethyl-4-methylenepyrrolidin-1-yloxy Radical (**3a**):

Yield: 630 mg (32 %); R_f 0.49 (hexane–EtOAc, 2:1) (TLC); oil.

2-Formylmethyl-2,2,6,6-tetramethyl-4-methylenepiperidin-1-yloxy Radical (**3b**):

Yield: 580 mg (27 %); R_f 0.42 (hexane–EtOAc, 2:1) (TLC); oil.

3-Ethoxycarbonylmethyl-2,2,6,6-tetramethyl-4-methylenepiperidin-1-yloxy Radical (4b):

A mixture of allylic alcohol **1b** (3.64 g, 20.0 mmol) triethyl orthoacetate (32.44 g, 0.2 mol) and propionic acid (0.148 g, 2.0 mmol) was heated at 140 °C with continuous removal of EtOH using a Dean–Stark apparatus. After 2 h, further propionic acid (0.148 g, 2.0 mmol) was added and the mixture was heated for 6 h. The excess of orthoacetate was then evaporated in vacuo. The red-brown residue was purified by flash chromatography (hexane–Et_2O) and gave ester **4b**. Yield: 3.05 g (60 %); R_f: 0.31 (hexane–Et_2O 2:1) (TLC); oil.

ω-Alkenyl Alcohols 5a,b; General Procedure:

Method A (from ω-Alkenyl Esters **4a,b***)*:

To a stirred refluxing solution of ω-alkenyl ester **4a,b** (20 mmol) and $NaBH_4$ (5.67 g, 0.15 mol) in anhydr. t-BuOH (30 mL), a mixture of anhydr. MeOH (8 mL) and t-BuOH (12 mL) was added dropwise over 3–4 h under N_2. Then the mixture was concentrated in vacuo, quenched with water (30 mL), extracted with $CHCl_3$ (3 × 20 mL), dried ($MgSO_4$), filtered and evaporated. The crude alcohol was purified by flash chromatography (hexane–EtOAc).

3-Hydroxyethyl-2,2,5,5-tetramethyl-4-methylenepyrrolidin-1-yloxy Radical (**5a**):

Yield: 2.57 g (65 %); R_f 0.31 ($CHCl_3$–Et_2O, 1:1) (TLC); oil.

3-Hydroxyethyl-2,2,6,6-tetramethyl-4-methylenepiperidin-1-yloxy Radical (**5b**):

Yield: 1.78 g (42 %); R_f 0.38 ($CHCl_3$–Et_2O, 1:1) (TLC); oil.

Method B (from ω-Alkenyl Aldehydes **3a,b***)*:

The mixture of ω-alkenyl aldehyde **3a,b** (10 mmol) and $NaBH_4$ (1.89 g, 50 mmol) in dry EtOH (30 mL) was stirred for 30 min at 40 °C. The mixture was then concentrated in vacuo, water (20 mL) was added, the mixture was extracted with chloroform, and the organic layer was washed with brine, dried ($MgSO_4$), filtered and evaporated.

3-Hydroxyethyl-2,2,5,5-tetramethyl-4-methylenepyrrolidin-1-yloxy Radical (**5a**):

Yield: 1.68 g (85 %); R_f 0.31 ($CHCl_3$–Et_2O, 1:1) (TLC); oil.

3-Hydroxyethyl-2,2,6,6-tetramethyl-4-methylenepiperidin-1-yloxy Radical (**5b**):

Yield: 1.69 g (80 %); R_f 0.38 ($CHCl_3$–Et_2O, 1:1) (TLC); oil.

3-Bromoethyl-2,2,5,5-tetramethyl-4-methylenepyrrolidin-1-yloxy Radical (6):

To a stirred solution of **5a** (2.97 g, 15 mmol) and Et_3N (1.62 g, 16 mmol) in dry CH_2Cl_2 (30 mL), methanesulfonyl chloride (1.83 g, 16 mmol) was added dropwise at 0 °C and the mixture was maintained at 0 °C for 1 h, then allowed to warm up to r.t. Water (10 mL) was added, the organic layer was separated and washed with 2 % aq HCl (10 mL), dried ($MgSO_4$), filtered and evaporated to dryness. The mixture of crude mesylate (3.93 g, 14.2 mmol) and LiBr (1.73 g, 20 mmol) was refluxed in dry acetone (30 mL) for 1 h. After filtration and rotary evaporation, the residue was dissolved in Et_2O, washed with water (20 mL), dried ($MgSO_4$), and evaporated. The residue was flash chromatographed (silica gel; hexane–Et_2O) to give the title compound **6**. Yield: 3.30 g (90 %); R_f 0.48 (hexane–Et_2O) (TLC); oil.

4-Azidophthalimide (8):

To a stirred solution of 4-aminophthalimide (8.10 g, 50 mmol) in conc. HCl (70 mL) and water (200 mL), $NaNO_2$ (3.79 g, 55 mmol) in water (15 mL) was added dropwise at 0 °C. The mixture was stired for 30 min, then NaN_3 (3.57 g, 55 mmol) in water (20 mL) was added slowly at 0 °C and the mixture was stirred for 30 min. The precipitate was filtered off, washed with water (50 mL), conc. HCl (100 mL), water (100 mL), and then dried in vacuum desiccator over P_2O_5 overnight.

Yield: 6.58 g (70 %); R_f 0.44 (hexane–Et_2O, 1:1) (TLC); mp: 179–180 °C.

3-[(4-Azidophthalimido)ethyl]-2,2,5,5-tetramethyl-4-methylenepyrrolidin-1-yloxy Radical (9):

A mixture of **6** (2.61 g, 10 mmol), 4-azidophthalimide (**8**) (1.88 g, 10 mmol), K_2CO_3 (1.38 g, 10 mmol), KOH (56 mg, 1 mmol) and 18-crown-6 (132 mg, 0.5 mmol) in dioxane (30 mL) was vigorously stirred and refluxed for 18 h. The mixture was filtered and the solvent was evaporated. The residue was dissolved in $CHCl_3$ (40 mL), washed with water (20 mL), dried ($MgSO_4$), and concentrated in vacuo. The brown residue was purified by flash chromatography (hexane–EtOAc).

Yield: 1.32 g (35 %); R_f 0.31 (hexane–EtOAc, 2:1) (TLC); mp: 110–112 °C.

^1H NMR (OH-form): $\delta = 7.76$ (d, 1 H, $J = 9$, 6′-H)*, 4.98, 5.08 (d, d 2 H, $J = 1.5$, $=CH_2$), 3.75 (m, 2 H, NCH_2), 2.22 (m, 1 H, 4-H), 1.8 (m, 2 H, $CH{-}CH_2$), 1.3 (s, 6 H, 2-CMe_2), 1.2, 0.95 (2 s, 2 × 3 H, 5-CMe_2).

1-Acetoxy-3-[(4-azidophthalimido)ethyl]-2,2,5,5-tetramethyl-4-methylenepyrrolidine-1-yloxy Radical (10):

To a solution of azidophthalimidyl radical **9** (1.0 g, 2.7 mmol) in dioxane (30 mL), a solution of ascorbic acid (1.76 g, 10 mmol) in water (10 mL) was added and the mixture was stirred at 40 °C for 15 min under N_2. The pale yellow solution was extracted with $CHCl_3$ (2 × 20 mL) and dried ($MgSO_4$) under N_2. First Et_3N (303 mg, 3 mmol) and then slowly AcCl (236 mg, 3 mmol) were added at 0 °C. The stirring was continued for 1 h, the mixture was filtered and the filtrate was evaporated to dryness. The residue was taken up in brine and extracted with $CHCl_3$ (3 × 20 mL). The organic layer was dried ($MgSO_4$), and evaporated to dryness. The residue was purified by flash chromatography (hexane–EtOAc) giving the diamagnetic azidophthalimidyl derivative **10**. Yield: 900 mg (81 %); R_f 0.36 (hexane–Et_2O, 1:1) (TLC); mp: 113–115 °C.

^1H NMR: $\delta = 7.85$ (d, 1 H, $J = 7.5$, 6′-H), 7.5 (d, 1 H, $J = 1$, 3′-H), 7.32 (dd, 1 H, $J = 7.5$, 1, 5-H), 5.15, 5.0 (d, d, 2 H, $J = 1.1$, CH_2), 3.8 (m, 2 H, NCH_2), 2.4 (m, 1 H, 4-CH), 2.2 (s, 3 H, $COCH_3$), 1.8 (m, 2 H, 4-CH_2), 1.3 (s, 6 H, 2-CMe_2), 1.15, 0.95 (s, bs, 6 H, 5-CMe_2).

1-Acetoxy-3-[(4-azidophthalimido)ethyl]-4-bromomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrole (11):

To a solution of the *O*-acetate **10** (1.0 g, 2.43 mmol) in anhydr. CCl_4 (20 mL) was added the brominating reagent NBS (534 mg, 3 mmol) or DDH (429 mg, 1.5 mmol) and benzoyl peroxide (14 mg, 0.1 mmol) and the mixture was refluxed for 14 h. The reaction was monitored by TLC (hexane–Et_2O, 1:1). The succinimide or 5,5-dimethylhydantoin was filtered off and washed with CCl_4 (10 mL). The filtrate was evaporated to dryness and flash chromatographed (hexane–EtOAc). Yield: 670 mg (56 %); R_f 0.33 (hexane–Et_2O, 1:1) (TLC); mp: 111–112 °C.

^1H NMR: $\delta = 7.85$ (d, 1 H, $J = 7.5$, 6′-H), 7.5 (d, 1 H, $J = 1$, 3′-H), 7.32 (dd, 1 H, $J = 7.5$, 1, 5′-H), 4.1 (m, 2 H, $BrCH_2$), 3.8 (m, 2 H, NCH_2), 2.5 (m, 2 H, 4-CH_2), 2.18 (s, 3 H, $COCH_3$), 1.3 (bs, 12 H, 2, 5-CMe_2).

3-[(4-Azidophthalimido)ethyl]-4-bromomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxy Radical (12):

The acetate **11** (980 mg, 2 mmol) was dissolved in THF (15 mL) and NaOMe solution, freshly made from dry MeOH (5 mL) and sodium (20 mg, 0.84 mmol), was added. The mixture was kept at r.t. for 30 min, then the solvents were evaporated, and the residue was dissolved in $CHCl_3$. The mixture was washed with brine, dried, filtered and evaporated. Purification of the crude mixture by flash chromatography (hexane–Et_2O) gave the pure radical. Yield: 250 mg (28 %); R_f 0.28 (hexane–Et_2O, 1:1) (TLC); mp: 130–132 °C.

3-[(4-Azido-*N*-phthalimido)ethyl]-4-methanethiosulfonylmethyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxy Radical (13):

The radical **12** (120 mg, 0.25 mmol) and $NaSSO_2Me$ (67 mg, 0.5 mmol) were dissolved in a mixture of dioxane (10 mL) and water (3 mL) and was refluxed for 30 min. The mixture was then concentrated in vacuo, the residue was dissolved in $CHCl_3$ (20 mL), washed with brine (10 mL), dried ($MgSO_4$), filtered, evaporated and purified by preparative TLC on a silica gel plate (hexane–EtOAc, 2:1). Yield: 78 mg (66 %); R_f 0.62 ($CHCl_3$–Et_2O, 1:1) (TLC); mp: 156–158 °C.

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* Further signals of the aromatic protons are obscured by the peaks of the diphenylhydrazine additive.

Table.

Product[a]	Yield (Method) (%)	mp (°C)	IR (neat or Nujol) ν (cm^{-1})	MS m/z (%)
2a	58	oil	1630, 1610 (C=C)	196 (M^+, 28), 153 ($M^+ - \cdot C_2H_3O$, 65), 138 ($153 - \cdot CH_3$, 100), 123 ($M^+ - \cdot C_2H_3O - \cdot NO$, 84), 81 (78), 69 (60), 41 (54)
2b	51	oil	1630, 1610 (C=C)	210 (M^+, 23), 167 ($M^+ - \cdot C_2H_3O$, 30), 137 ($M^+ - \cdot C_2H_3O - \cdot NO$, 34), 95 (77), 81 (100), 41 (34)
3a	32	oil	1720 (aldehyde C=O), 1650 (C=C)	196 (M^+, 85), 154 ($M^+ - \cdot CHCHO$, 100), 153 ($M^+ - \cdot CH_2CHO$, 68), 138 ($153 - \cdot CH_3$, 84), 123 ($153 - \cdot NO$, 52)
3b	27	oil	1710 (aldehyde C=O), 1640 (C=C)	210 (M^+, 21), 168 ($M^+ - \cdot CHCHO$, 9), 137 ($M^+ - \cdot CH_2CHO - \cdot NO$, 32), 109 (37), 74 (100), 56 (77)
4b	60	oil	1730 (ester C=O), 1650 (C=C)	254 (M^+, 1.2), 226 ($M^+ - \cdot C_2H_4$, 6.5), 195 (50), 167 (32), 135 (84), 121 (96), 107 (100), 93 (70), 74 (87), 56 (58), 41 (48)
5a	65 (A) 85 (B)	oil	3400 (OH), 1660 (C=C)	198 (M^+, 68), 153 [$M^+ - \cdot (CH_2)_2OH$, 16], 121 (38), 109 (67), 107 (95), 95 (59), 81 (58), 74 (39), 69 (81), 67 (78), 56 (56), 41 (100)
5b	42 (A) 80 (B)	oil	3340 (OH), 1640 (C=C)	212 (M^+, 18), 152 (34), 123 (40), 109 (35), 81 (62), 74 (100), 56 (37)
6	90	oil	1610 (C=C)	260/262 (M^+, 17/17), 151 (50), 95 (100), 41 (41)
8	70	179–180	3200 (NH), 2120 (N_3), 1760, 1720 (C=O)	188 (M^+, 12), 160 ($M^+ - N_2$, 72), 117 ($M^+ - N_2 - HNCO$, 46), 89 $117 - CO$, 100), 62 ($89 - HCN$, 37)
9	35	110–112	2110 (N_3), 1760, 1700 (C=O), 1605 (C=C)	368 (M^+, 33), 340 ($M^+ - N_2$, 31), 150 (47), 135 (100), 107 (83), 41 (59)
10	81	113–115	2100 (N_3), 1760, 1710 (C=O), 1610 (C=C)	411 (M^+, 1.5), 369 ($M^+ - CH_2CO$, 36), 354 ($M^+ - CH_2CO - \cdot CH_3$, 90), 326 ($354 - N_2$, 100), 107 (41)
11	56	111–112	2120 (N_3), 1760, 1700 (C=O), 1605 (C=C)	489/491 (M^+, 0.5/0.5), 474/476 ($M^+ - \cdot CH_3$, 59/58), 447/449 ($M^+ - CH_2CO$, 18/17), 432/434 ($M^+ - CH_2CO - \cdot CH_3$, 87/87), 404/406 ($432/434 - N_2$, 84/85), 43 (100)
12	28	130–132	2105 (N_3), 1760, 1700 (C=O), 1605 (C=C)	446/448 (M^+, 18/18), 418/420 ($M^+ - N_2$, 23/22), 367 ($M^+ - \cdot Br$, 42), 352 ($M^+ - \cdot Br - \cdot CH_3$, 40), 324 ($352 - N_2$, 42), 323 ($M^+ - N_2 - \cdot CH_3 - HBr$, 38), 153 (100)
13	66	156–158	2110 (N_3), 1760, 1700 (C=O), 1605 (C=C)	478 (M^+, 60), 450 ($M^+ - N_2$, 30), 448 ($M^+ - \cdot NO$, 100), 420 ($M^+ - \cdot NO - N_2$, 83), 352 ($M^+ - \cdot CH_3$, $-SSO_2CH_3$, 41), 164 (60), 117 (70)

[a] All compounds gave C, H, N analysis ± 0.21 %.

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