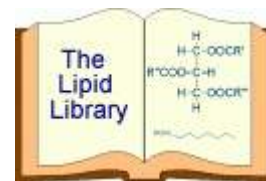


## FATTY ACIDS AND MASS SPECTROMETRY

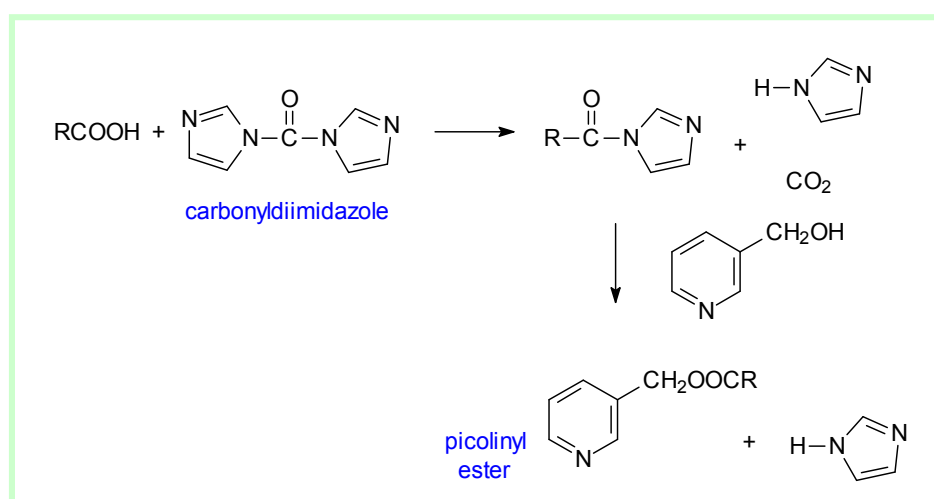
# Preparation of Nitrogen-Containing Derivatives for Mass Spectrometry of Fatty Acids



### 1. Principles

Following a brief description of the methodology, practical details are given here for preparation of the nitrogen-containing derivatives of fatty acids, i.e. picolinyl esters, 4,4-dimethyloxazoline (DMOX) derivatives and pyrrolidides, which are most useful for mass spectrometric analysis. Preparation of methyl ester derivatives is described on a separate webpage. Other useful derivatization techniques, e.g. hydrogenation, deuteration, *etc.*, are described in the section of these pages dealing with “**Mass spectrometry of methyl esters – further derivatization**”.

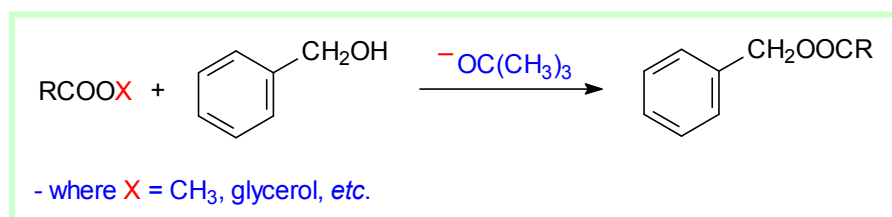
Until recently, picolinyl esters were prepared from free fatty acids only, so intact lipid or methyl ester samples were first hydrolysed. The author has then used a mild quantitative method developed for derivatizing sensitive polyunsaturated fatty acids containing epoxy groups involving an imidazolid intermediate [1]. It is simple and rapid, involving brief reactions with first carbonyldiimidazole then 3-(hydroxymethyl)pyridine and a catalyst.



The first method applied to prepare picolinyl esters was a rapid reaction with thionyl chloride to prepare the acid chloride, followed by reaction with 3-hydroxymethylpyridine (3-pyridylcarbinol) in acetonitrile [2]. The reaction is simple and rapid, but could be harmful to sensitive fatty acids, such as polyunsaturates. Acid chlorides can also be prepared by the milder method of reaction with oxalyl chloride overnight [3]. Dry solvents and fresh reagents are required because the reactions are sensitive to moisture.

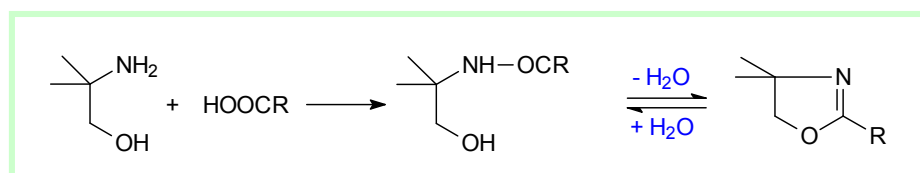
It has now been shown that picolinyl esters can be prepared directly from intact lipids or methyl esters by transesterification with 3-hydroxymethylpyridine catalysed by potassium *tert*-butoxide in tetrahydrofuran [4]. This reaction may indeed be useful for the preparation of a variety of different ester derivatives by substituting the appropriate alcohol. However, the reagent must be very dry, otherwise a competing irreversible hydrolysis reaction can occur. For this reason, I have had poor

results with this method, and I have not had an opportunity to explore the reaction further to see if this deficiency can be overcome.



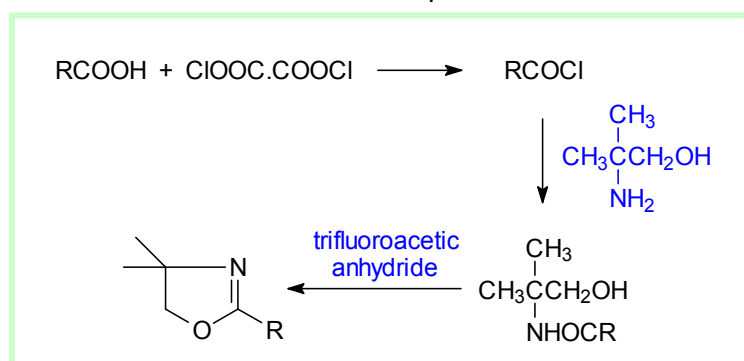
Picolinyl esters can be converted back to methyl esters by conventional acidic or basic transesterification procedures.

DMOX derivatives are prepared in a simple one-pot reaction by reaction of lipids with 2-amino-2-methyl-1-propanol in a nitrogen atmosphere at 180°C (a minimum of 2 hours for free acids, or 18 hours for methyl esters and intact lipids [5]).



Unfortunately, we have occasionally observed incomplete reaction and appearance of an intermediate that elutes later from GC columns (and gives a mass spectrum almost identical to that of the required derivative). It is important to keep the product and reaction mixtures dry, otherwise traces of moisture can cause ring opening, even on storage in inert solvents at low temperatures [6]. For the same reason, it is not possible to purify DMOX derivatives by adsorption chromatography.

The prolonged high temperature required for the preparation of DMOX derivatives gives cause for concern, and there must be some risk to polyunsaturated fatty acids or any other compound with a labile functional group. For example, *trans*-3-hexadecenoic acid, common in plant photosynthetic tissue, was found to have isomerized largely to *cis*-2-hexadecenoic acid during the reaction [7], and crepenynic acid was found to undergo a cyclization reaction [8]. We have also observed that linoleic acid can be partially isomerized to conjugated isomers ('CLA') if the reaction is carried out carelessly. An alternative two-step reaction has been described that may be safer in some circumstances [8]. It involves preparing the simple amide via the acid chloride, followed by cyclization with trifluoroacetic anhydride. As with picolinyl esters, I prefer to prepare DMOX derivatives via the free acids rather than from intact lipids.

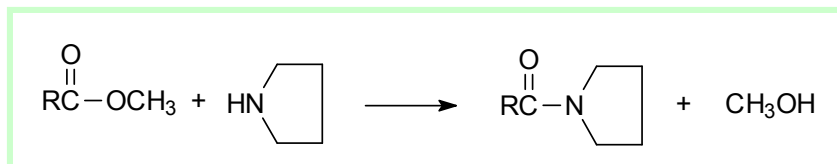


A further two-step procedure that is suitable for the preparation of both DMOX and simple oxazoline derivatives has been described, and the GC-MS properties of the latter appear excellent [9]. However, few published spectra are available. Of the many alternative nitrogen-containing

derivatives for mass spectrometry that have been described in the literature, this appears to me to be the only one that might be worth pursuing. Similarly, a new mild procedure for preparing DMOX derivatives and pyrrolidides from free acids, which should also be applicable to picolinyl esters, has recently been described [10,11]. We have yet to try it out so would be interested in learning of the experience of others.

DMOX derivatives can be converted back to methyl esters by methylation procedures used for sphingolipids, e.g. by reaction with methanol containing hydrochloric acid [6].

Pyrrolidides are prepared simply by reacting methyl esters of fatty acids with pyrrolidine in the presence of acetic acid [12].



The reaction takes place under basic conditions and is relatively mild so may be safer than for DMOX derivatives with fatty acids containing labile functional groups. Procedures are also available to prepare pyrrolidides directly from triacylglycerols and presumably other glycerolipids [13] or from free fatty acids [10,14,15], the latter in a mild one-pot reaction.

## 2. Practical Procedures

### Laboratory protocol for hydrolysis of lipids [6]:

A solution of 0.1M potassium hydroxide in 90% aqueous ethanol is prepared immediately before use by adding 1 volume of standard 1M aqueous potassium hydroxide to 9 volumes of ethanol. To the lipid sample (up to 6 mg) in a stoppered centrifuge tube is added 0.1M potassium hydroxide in 90% aqueous ethanol (0.25 mL per mg sample). Leave at 50°C in a water bath or heating block for 3 hours. Remove from water bath and add 2M hydrochloric acid (0.05 mL per mg sample) using a Pasteur pipette. Add isohexane (3 mL), diethyl ether (3 mL) and distilled water (2 mL). Shake thoroughly and, if necessary (if there are not two distinct layers and/or an emulsion is formed), centrifuge for 5 min (about 400 rpm). Put upper organic layer through a short (3 cm) column of anhydrous sodium sulfate (prepared in a Pasteur pipette, plugged with a small piece of cotton wool, and pre-washed with 3 mL isohexane-diethyl ether (1:1, by vol.) prior to use) to dry. Collect in a test tube. Repeat extraction of lower aqueous layer with isohexane-diethyl ether (3 mL, 1:1, by vol.) and put the organic layer through the sodium sulfate column. Wash sample through the column into the tube with more isohexane-diethyl ether (3 mL). Take sample to dryness in a gentle stream of nitrogen on a heating block at 30°C

### Laboratory protocol for preparation of picolinyl esters via an imidazole intermediate [1]:

The reaction, as detailed below, is moisture sensitive and all reagents should be protected from moisture (*i.e.* by storing in the presence of a desiccant). The carbonyldiimidazole solution, in particular, should be freshly made up each time a set of samples are derivatized. The picolinyl derivatizing reagent is prepared by dissolving

3-(hydroxymethyl)pyridine (900  $\mu\text{L}$ ) in dichloromethane (9 mL) and triethylamine (9 mL), and adding a little anhydrous sodium sulfate (2-3 mm in tube). It can be used for up to one month if kept in a sealed tube (with PTFE-lined screw top) in the refrigerator. The free fatty acid sample (up to 1 mg), in a centrifuge tube, is dissolved in dichloromethane (100  $\mu\text{L}$ ) and a solution of 1,1'-carbonyldiimidazole (100  $\mu\text{L}$ , 10 mg/mL in dichloromethane, freshly prepared) is added. Leave 1 min (not longer) at room temperature before proceeding to the next step. Add picolinyl reagent (200  $\mu\text{L}$ ). Agitate and leave for 10 min. at 37°C. At the end of this time add acetic acid (25  $\mu\text{L}$ ). Take to dryness on a heating block at 30°C with a gentle stream of nitrogen. Add isohexane (5 mL) and distilled water (2 mL). Shake thoroughly and vortex. Put upper isohexane layer through a short (4 cm) column of anhydrous sodium sulfate in a Pasteur pipette (with a small cotton wool plug and pre-washed with isohexane) to dry. Re-extract the aqueous layer with fresh isohexane (2 mL), and pass through the column also. If necessary, a little crystalline potassium chloride can be added to break emulsions. Finally, wash through column with isohexane (1 mL) and evaporate the combined isohexane eluents. Dissolve sample in an appropriate amount of isohexane for GC and GC-MS analyses.

#### Laboratory protocol for preparation of picolinyl esters via transesterification [4]:

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.1 mL, 1.0 M) is added to 3-(hydroxymethyl)pyridine (0.2 mL). After mixing, the lipid sample (up to 10 mg) in dry dichloromethane (1 mL) is added, and the mixture is held at 40°C for 30 min in a closed vial. After cooling to room temperature, water (2 mL) and hexane (4 mL) are added, and the organic phase is collected, dried over anhydrous sodium sulfate, and evaporated. The sample is dissolved in isohexane for GC-MS analysis.

#### Laboratory protocol for preparation of picolinyl esters via acid chlorides [2]:

All reactions are carried out in 0.3 ml screw cap vials. The acid (20  $\mu\text{g}$ ) is heated with thionyl chloride (20  $\mu\text{L}$ ) for 10 min. at 100°C. The thionyl chloride is evaporated in a stream of nitrogen, a solution of 20% 3-(hydroxy-methyl)pyridine in acetonitrile (10  $\mu\text{L}$ ) is added, and the mixture heated for 1 min. at 100°C. An aliquot is injected directly onto the GC column.

#### Laboratory protocol for preparation of DMOX derivatives by the one-step reaction [5]:

To the lipid sample (up to 2 mg) in a test tube is added 2-amino-2-methyl-1-propanol (0.25 g). The vessel is flushed with nitrogen, stoppered, and placed in a heating block, at 190°C overnight. Flush the tube with nitrogen when the tube is up to temperature to help to eliminate moisture and minimize autoxidation. A little glycerol added to the well of the heating block ensures good contact and that the correct temperature is maintained. Next day, remove the test tube from the heating block, allow to cool to room temperature and wash off any glycerol on the external surface with water. Add diethyl ether-isohexane (1:1, v/v; 5 mL) to the tube, washing down the internal surface,

followed by water (5 mL). Shake thoroughly and allow layers to settle. If necessary, addition of a little sodium or potassium chloride will usually break up any interfacial layers. Transfer the organic layer with a pasteur pipette to a fresh test-tube, and re-extract the aqueous layer with fresh solvent (2 mL). Add distilled water (3 mL) to the combined solvent layers, shake and allow to settle. Transfer the solvent layer to a fresh tube with a pasteur pipette and add anhydrous sodium sulfate (about 1 mL). Leave for one hour with occasional vigorous shaking, then pass solvent layer through a short (3 cm) column of anhydrous sodium sulfate (prepared in a Pasteur pipette, plugged with a small piece of cotton wool, and pre-washed with ca. 3 mL isohexane prior to use) to dry, into a fresh test tube. Wash sample from the sodium sulfate tube through column with isohexane (2 mL). Take sample to dryness in a gentle stream of nitrogen on a heating block at 30°C. Dissolve the sample in an appropriate amount of isohexane for GC and GC-MS analyses. Adding a few crystals of anhydrous sodium sulfate stabilizes the derivative for storage.

Note that thorough drying in this way gives a more stable product. All steps should be carried out as expeditiously as possible. (Note that DMOX derivatives are not easily purified by adsorption chromatography).

#### Laboratory protocol for preparation of DMOX derivatives by the two step reaction [8]:

The fatty acid must first be converted to the acid chloride as in the method for preparation of picolinyl esters via the acid chloride. This is used immediately for the preparation of the required derivatives.

A solution of 2-amino-2-methylpropanol in dichloromethane (10 mg/mL) is prepared and stored over anhydrous sodium sulfate. It is stable in the refrigerator for about 1 month. This solution (0.5 mL) is added to the freshly prepared acid chloride, cooled in an ice bath, and the mixture is then left to warm up to room temperature for an hour. The solvent is removed in a stream of nitrogen, and trifluoroacetic anhydride (0.5 mL) is added. After 1 hour on a heating block at 50°C, or 3 hours at room temperature, the reagent is removed in a stream of nitrogen. Isohexane (5 mL) is added followed by water (2 mL), and the product is obtained as in the previous reaction, taking care to dry the product thoroughly.

Note that compounds that appear to be closely related to the DMOX derivatives but eluting a little later on GC can be 1-2% of the total, and at the moment we have no method of eliminating these.

#### Laboratory protocol for preparation of pyrrolidide derivatives from fatty acid methyl esters [12]:

The fatty acid methyl ester (up to 10 mg) is dissolved in freshly distilled pyrrolidine (1 mL), acetic acid (0.1 mL) is added, and the mixture is heated at 100°C for 1 hour. Excess pyrrolidine is evaporated in a stream of nitrogen at 50°C, and then the residue is taken up in hexane-diethyl ether (1:1, v/v; 8 mL) and is washed three times with water (4 mL portions). After drying over anhydrous sodium sulfate, the required product is obtained on evaporation of the solvent.

## References

1. Balazy, M. and Nies, A.S. Characterization of epoxides of polyunsaturated fatty acids by mass spectrometry via 3-pyridinylmethyl esters. *Biomed. Environ. Mass Spectrom.*, **18**, 328-336 (1989).
2. Harvey, D.J. Picolinyl esters as derivatives for the structural determination of long chain branched and unsaturated fatty acids. *Biomed. Mass Spectrom.*, **9**, 33-38 (1982).
3. Mattson, F.H. and Volpenhein, R.A. Synthesis and properties of glycerides. *J. Lipid Res.*, **3**, 281-296 (1962).
4. Destailats, F. and Angers, P. One-step methodology for the synthesis of FA picolinyl esters from intact lipids. *J. Am. Oil Chem. Soc.*, **79**, 253-256 (2002).
5. Fay, L. and Richli, U. Location of double bonds in polyunsaturated fatty acids by gas chromatography-mass spectrometry after 4,4-dimethyloxazoline derivatization. *J. Chromatogr. A*, **541**, 89-98 (1991).
6. Christie, W.W. *Lipid Analysis (3rd edition)* (Oily Press, Bridgwater) (2003).
7. Lamberto, M. and Ackman, R.G. Positional isomerization of *trans*-3-hexadecenoic acid employing 2-amino-2-methylpropanol as a derivatizing agent for double bond location by GC/MS. *Anal. Biochem.*, **230**, 224-228 (1995).
8. Christie, W.W. Mass spectrometry of fatty acids with methylene-interrupted ene-yne systems. *Chem. Phys. Lipids*, **94**, 35-41 (1998).
9. Kuklev, D.V. and Smith, W.L. A procedure for preparing oxazolines of highly unsaturated fatty acids to determine double bond positions by mass spectrometry. *J. Lipid Res.*, **44**, 1060-1066 (2003).
10. Kangani, C.O. and Kelley, D.E. One pot direct synthesis of amides or oxazolines from carboxylic acids using Deoxo-Fluor reagent. *Tetrahedron Letts.*, **46**, 8917-8920 (2005).
11. Kangani, C.O., Kelley, D.E. and Evans, R.W. Synthesis and mass spectrometry of benzoxazoline, dimethyloxazoline and 4-phenyloxazoline derivatives of polyunsaturated fatty acids. *Rapid Commun. Mass Spectrom.*, **21**, 2129-2136 (2007).
12. Andersson, B.A. and Holman, R.T. Pyrrolidides for mass spectrometric determination of the position of double bonds in monounsaturated fatty acids. *Lipids*, **9**, 185-190 (1974).
13. Vetter, W. and Walther, W. Pyrrolidides as derivatives for the determination of the fatty acids of triacylglycerols by gas-chromatography. *J. Chromatogr. A*, **686**, 149-154 (1994).
14. Vetter, W. and Walther, W. Preparation of pyrrolidides from fatty acids via trimethylsilyl esters for GC-MS analysis. *J. Chromatogr. A*, **513**, 405-407 (1990).
15. Kangani, C.O., Kelley, D.E. and DeLany, J.P. New method for GC/FID and GC-C-IRMS analysis of plasma free fatty acid concentration and isotopic enrichment. *J. Chromatogr. B*, **873**, 95-101 (2008).

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Last updated: 15/6/2008

