Gas and High Pressure Liquid Chromatographic Properties of Some 4-Nitrobenzamides of Amphetamines and Related Arylalkylamines

C. Randall Clark,* J. Dwayne Teague, Martha M. Wells, and Joel H. Ellis

School of Pharmacy, Auburn University, Auburn, Alabama 36830

Amphetamines and related arylalkylamines were converted in high yields to the corresponding 4-nitrobenzamides (4-NBA) to enhance their UV detectability. The derivatives are chemically stable and can be rapidly prepared and purified by extraction. These amides were separated by reverse phase high pressure liquid chromatography using an isocratic solvent system. The molar absorptivity of amphetamine 4-NBA is 1.2 $\times 10^4$ L mol^{-1} cm^{-1} at 254 nm which results in a 20-ng sample yielding a peak 5% of scale. The 4-NBA derivatives exhibited excellent gas chromatographic properties on 3% OV-17 liquid phase. The amides are much less volatile than the parent amines requiring column temperatures in excess of 240 °C for elution. The derivatives are thermally stable at these temperatures as shown by chemical ionization mass spectrometry. Thus, arylalkylamines can be derivatized, the 4-NBA derivative analyzed by HPLC, and the sample collected from the effluent and subjected to GC-MS analysis for the generation of additional analytical data.

High pressure liquid chromatography has become a very valuable technique in the analysis of drugs from pharmaceutical formulations (*1*, *2*) and biological samples (*3*, *4*). Sunshine et al. (*5*) recently reviewed the relative merits of some methods for amphetamine assay in biological fluids. The analysis of amphetamines and other arylalkylamines by high pressure liquid chromatography is limited by the relatively low absorptivity of this class of compounds. However, the use of HPLC in the analysis of amphetamines in dosage forms has been reported (*6*). The excellent separation powers and the nondestructive nature of spectrophotometric detection makes HPLC an ideal method of analysis for amphetamines and related amines in biological samples. The molar absorptivity of amphetamine (*7*) in 0.1 N sulfuric acid is 202 L mol^{-1} cm^{-1} which limits the use of spectrophotometric detection of therapeutic levels. Subtherapeutic levels are reported (*8*) to be 0.1 μg/mL or less and, in order to achieve this level of sensitivity in HPLC, the arylalkylamines must be derivatized with a strong chromophoric group. The necessity of derivatization for detectibility in HPLC has been described by Jupille (*9*).

The 4-nitrobenzoyl group has been shown to impart high absorptivity to other classes of compounds (*10*). Experience with 4-NBA derivatives has indicated their relative ease of formation and good chemical stability. These features should allow for the development of a rapid and sensitive method for the detection of arylalkylamines by HPLC. The nondestructive spectrophotometric method of detection in HPLC is an additional advantage in the analysis of samples of limited quantity. This allows for sample collection following analysis and the generation of additional analytical data from the sample; however, these data must be generated on the derivatized sample.

The purpose of this paper is to report a study of the preparation and chromatographic properties (HPLC and GLC) of a representative sample of arylalkylamine 4-nitrobenzamides. A rapid method for derivatizing amines in aqueous solution was developed utilizing a water-tetrahydrofuran solvent system. Separation of the amides by HPLC was achieved using reverse phase chromatography with an isocratic solvent system consisting of water and acetonitrile. Studies on the GLC properties of the amides showed these compounds to be suitable for analysis by this method. The chemical ionization mass spectra of the amides were consistent with the assigned structures.

EXPERIMENTAL

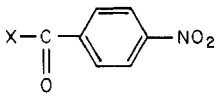
Apparatus. The liquid chromatograph consisted of a Waters model 6000 solvent pump, model U6K injector equipped with a 2-mL loop, model 440 UV detector, and a Varian model A-25 recorder. UV measurements were carried out on a Beckman Acta VI or a Hitachi 60 double beam spectrophotometer. IR spectra were determined on a Beckman 4230 spectrophotometer and NMR spectra were recorded using a Varian T-60A instrument. Gas chromatography–mass spectrometry was performed using a Finnigan 9500 gas chromatograph coupled with a Finnigan 3300 mass spectrometer. The mass spectrometer ion current integrator served as the detector for the gas chromatograph. Chemical ionization mass spectra were obtained using methane (1 Torr) as the reagent gas. Reference compounds were analyzed by direct probe insertion into the ionizer region of the mass spectrometer for comparison with spectra obtained by GC-MS.

Reagents and Chemicals. All reagents were used as purchased without further purification. Amphetamine sulfate and methamphetamine hydrochloride were obtained from Sigma Chemicals Co., St. Louis, Mo. The 4-nitrobenzoylchloride, *n*-propylamine, benzylamine, α-methylbenzylamine, phenethylamine, and spectrophotometric grade methanol were purchased from Aldrich Chemical Co., Milwaukee, Wis. Spectrophotometric grade acetonitrile was obtained from Burdick and Jackson Laboratories, Muskegon, Mich. *N*-*n*-Propylamphetamine (1-phenyl-2-(*n*-propylamino) propane) was prepared by a procedure similar to that of Brackett et al. (*11*).

Synthesis of 4-Nitrobenzamides. Reference standards of the 4-nitrobenzamide derivatives were prepared according to the following general procedure. A solution of the amine or amine salt (0.017 mol) and triethylamine (0.034 mol) in 150 mL of tetrahydrofuran was added to a 500-mL 3-necked flask. A solution of 4-nitrobenzoylchloride (0.034 mol) in 50 mL of tetrahydrofuran was added dropwise and the mixture refluxed for 4 h. The excess acid chloride was hydrolyzed by the addition of water and the organic phase was evaporated. The residue was taken up in 100 mL of chloroform and extracted with a solution of 10% potassium carbonate (3 × 50 mL), then washed with 50 mL of water. The chloroform was dried ($MgSO_4$) and evaporated. The compounds were recrystallized from a benzene–hexane solution to yield white crystalline solids. The molar absorptivity of each amide was determined in absolute ethanol.

Derivative Formation. To a 5-mL reaction vial was added 1 mL of an aqueous amine solution (300 to 20 μg/mL), 2 mL of a freshly prepared 4-nitrobenzoylchloride solution in tetrahydrofuran (50 mg/mL), and 1 mL of 1 M sodium hydroxide. The vial was sealed with a Teflon-lined screwcap and placed in a water bath at 65 °C for 1 h. The derivatives can be chromatographed directly or further purified according to the following procedure. The contents of the vial were added to a 125-mL separatory funnel

Table I. Absorptivity and Chromatographic Properties of the 4-NBA Derivatives



Compound No.	X	E(254 nm) × 10^3	$E(\lambda_{max})$ × 10^3	Relative elution order	
				C_{18}	OV-17
1	$CH_3CH_2CH_2NH$	11.4	12.9 (265)	1	1
2	$C_6H_5CH_2NH$	12.1	13.7 (265)	2	3
3	$C_6H_5CHCH_3NH$	11.2	12.5 (265)	3	2
4	$C_6H_5CH_2CH_2NH$	11.5	13.1 (265)	3	7
5	$C_6H_5CH_2CHCH_3NH$	12.3	14.2 (265)	4	4
6	$C_6H_5CH_2CHCH_3NCH_3$	7.9	10.5 (271)	5	5
7	$C_6H_5CH_2CHCH_3NCH_2CH_2CH_3$	8.3	10.7 (274)	6	6

and the pH adjusted to 12 by the addition of 1 M sodium hydroxide. The solution was extracted with chloroform (2 × 10 mL) and the extracts combined and washed with 10% potassium carbonate (2 × 20 mL), then water. The organic phase was dried ($MgSO_4$), evaporated under a stream of air, and the residue taken up in a known quantity of methanol.

The yield of this process was studied using standard solutions containing various amounts of amphetamine. The derivative formation was carried out on 300, 100, and 20 μg of amphetamine sulfate.

Chromatographic Procedure. Separation by high pressure liquid chromatography was accomplished using a $^1/_4$-in. o.d. by 30 cm μ-Bondapak C_{18} column (Waters Associates, Milford, Mass.). The mobile phase consisted of a mixture of 65% water (double distilled) and 35% acetonitrile. The mobile phase flow rate was 1.5 mL/min, and the UV detector was operated at 254 nm. In all runs, 5 μL of a methanol solution of the amides was injected using a 25-μL syringe. The separations were carried out at ~20 °C (room temperature) without thermostating.

The gas chromatograph was equipped with a 2-mm i.d. by 5-ft glass column, the carrier gas (methane) flow rate was approximately 20 mL/min. Compounds were chromatographed on 3% OV-1 on 80/100 mesh chromosorb (Perkin-Elmer Corp. Norwalk, Conn.), 3% OV-17 on 80/100 mesh Chromosorb, and 3% OV-22 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.). The gas chromatograph was operated at the following conditions: injector 290 °C, column oven 255 °C, separator and transfer line 290 °C. The mass spectrometer analyzer temperature was 50 °C.

RESULTS AND DISCUSSION

Our initial studies reported here involve the formation of the 4-nitrobenzamide (4-NBA) derivatives of amphetamine, methamphetamine, and other related amines, the synthesis of reference samples, and a study of the HPLC and GC-CIMS properties of these amides. The reference materials were prepared by reacting 4-nitrobenzoylchloride with the appropriate amine or amine salt in the presence of base. The amides were purified by recrystallization and showed characteristic IR and NMR spectra. The UV data for these compounds are reported in Table I. Molar absorptivity values were calculated at the wavelength of maximum absorbance and at 254 nm (wavelength used in most fixed wavelength UV detectors for HPLC).

The derivatization process was carried out in a tetrahydrofuran–water solvent system. The procedure allowed for the rapid and direct derivatization of an amine from aqueous solution. Thus, no previous sample preparation is necessary. The attempted isolation of such amines from an aqueous solution is reported to result in almost complete loss of sample (*12*). The derivatization mixture can be chromatographed directly for high concentrations of amines. However, for low amine levels, a sample cleanup by solvent extraction was required. The amides are neutral species in an acid–base extraction scheme and are much less volatile compounds than the starting amines. Therefore, sample loss due to vaporization is not a problem with the derivatized samples.

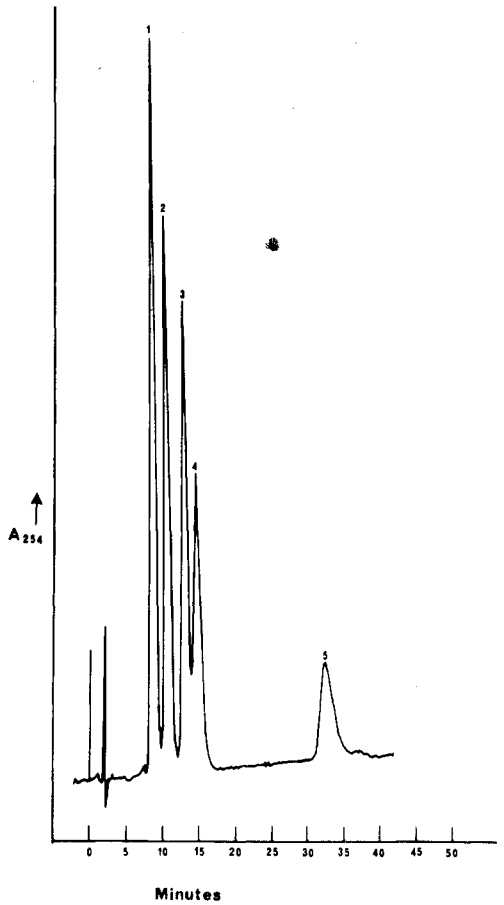


Figure 1. Water–acetonitrile isocratic elution HPLC of arylalkyl 4-NBA derivatives. Peak: (1) benzylamine, (2) phenethylamine (or α-methylbenzylamine), (3) amphetamine, (4) methamphetamine, (5) *n*-propylamphetamine

The amides are strong absorbers at 254 nm as indicated by the molar absorptivities in Table I. This absorption makes for good detectability. Thus, a 20-ng injection of amphetamine 4-NBA gave a peak 5% of full scale at a 5:1 signal to noise ratio. Figure 1 shows the separation of the arylalkyl 4-NBA derivatives using an isocratic solvent system consisting of 65% water–35% acetonitrile. Similar separations were obtained with a 1:1 water–methanol solvent system. The retention of these amides by the C_{18} reverse phase column increases with increasing molecular weight. This system failed to separate the 4-NBA derivatives of phenethylamine and α-methylbenzylamine (isomeric compounds with the same molecular weight). The separation time of 35 min for the isocratic system can be reduced through solvent programming. Peak area vs. concentration plots were linear for all compounds studied in this project. The quantitative applicability of this method

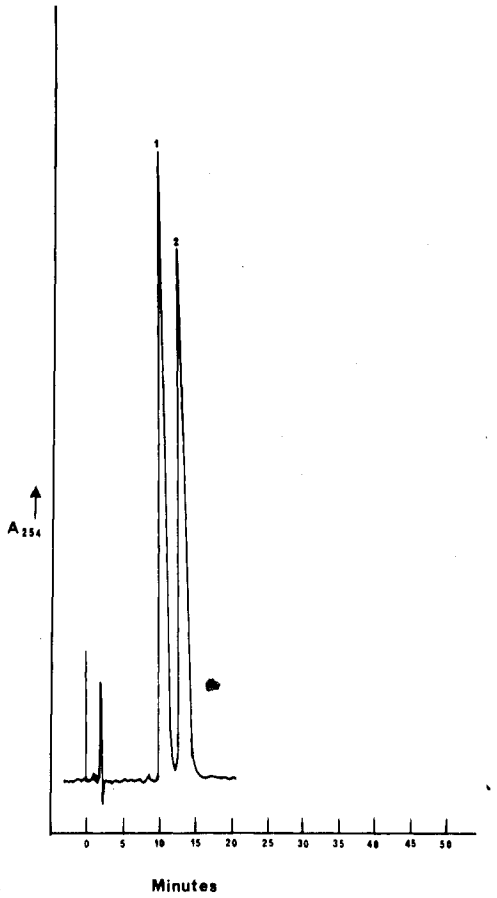


Figure 2. HPLC separation of phenethylamine 4-NBA (1) and amphetamine 4-NBA (2)

was examined by a study of the yields of amides at various amine concentrations. Amphetamine sulfate solutions were used in this study. The average yield of 4-NBA isolated following solvent extraction was 86%, $P(0.05) = 86\% \pm 3\%$.

Derivatization is often required to produce good gas chromatographic properties in amphetamines. The final phase of this preliminary investigation was to examine the gas chromatographic properties of the 4-nitrobenzamides. The amides are much less volatile than the parent amines and require column oven temperature of 240 °C or higher for gas chromatography. The amides were chromatographed on OV-1, OV-17, and OV-22 stationary phases. Satisfactory separation was obtained only on the 3% OV-17 column. Although the OV-17 column gave better separation, the retention times were very close for many of these compounds. The elution order reported in Table I was determined by mass spectral analysis of the partially resolved peaks following injection of a solution containing a mixture of compounds 1–7. The gas chromatographic properties of the 4-NBA derivatives can best be described by dividing the compounds into two groups: 1) straight chain arylalkylamines and 2) α-methyl arylalkylamines. The elution time on OV-17 increases with increasing molecular weight in each series of compounds. The α-methylamine 4-NBA derivative has a shorter retention time than its desmethyl straight chain analogue: The 4-NBA of amphetamine elutes before phenethylamine and α-methylbenzylamine has a shorter retention time than benzylamine 4-NBA. The isomeric 4-NBA derivatives of phenethylamine and α-methylbenzylamine not separated by HPLC were easily separated by the OV-17 column, the α-methylbenzylamine 4-NBA having the shorter retention time. The shortened retention times of the α-methylamine derivatives caused the peaks for amphetamine and benzylamine to overlap and this accounts for the partial resolution of the mixture. Analysis

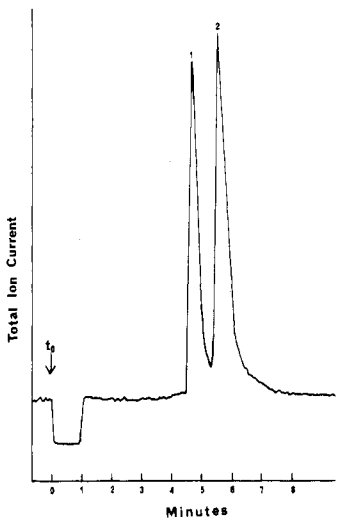


Figure 3. GC separation of amphetamine 4-NBA (1) and phenethylamine 4-NBA (2)

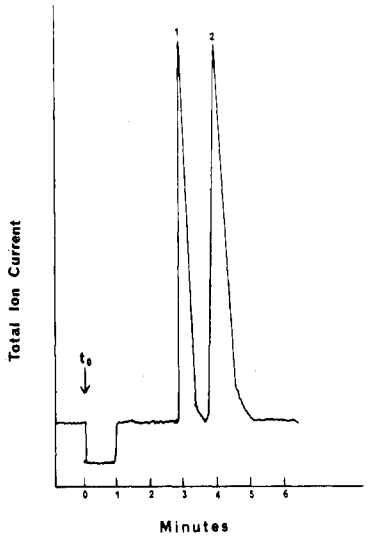


Figure 4. GC separation of α-methylbenzylamine 4-NBA (1) and amphetamine 4-NBA (2)

of these preliminary data indicates that the derivatization procedure brings advantages to HPLC and that the derivatives can still be separated by GC to yield additional data.

Both phenethylamine (*8*) and *n*-propylamphetamine (*10*) have been used as internal standards for gas chromatographic analysis of amphetamines. An examination of the chromatographic properties of these compounds shows the 4-NBA of phenethylamine to be a better internal standard for this method. The internal standard used in HPLC should have an absorptivity in the range of the amphetamine 4-NBA. Therefore, the internal standard must be an amine of the same degree of substitution. Phenethylamine or α-methylbenzylamine are well suited as an internal standard for this method. The separation of phenethylamine and amphetamine 4-NBA by HPLC and GC are shown in Figures 2 and 3. Good separation was obtained in each method; however, the elution order is reversed. The derivatized amphetamine elutes first in the GC separation and second in the HPLC separation. Similar separations are obtained with α-methylbenzylamine; however the elution order is the same in HPLC and GC (Figure 4).

The chemical ionization mass spectra of the amides showed major ions at M + 1, M + 29, and M + 41 as expected. The only major fragmentation (M − 91) occurred with tertiary amides 6 and 7 and resulted from the loss of the benzyl group.

LITERATURE CITED

(1) E. Heftmann, Ed., "Chromatography, Third Ed.", Van Nostrand Reinhold Company, New York, N.Y., 1975, pp 675–714.
(2) C. K. Wong, D. M. Cohen, and K. P. Munnelly, *J. Pharm. Sci.*, **65**, 1090 (1976).
(3) M. J. Cooper, A. R. Sinaiko, M. W. Anders, and B. L. Mirkin, *Anal. Chem.*, **48**, 1110 (1976).
(4) L. C. Franconi, G. L. Hawk, B. J. Sandmann, and W. G. Haney, *Anal. Chem.*, **48**, 372 (1976).
(5) R. O. Bost, C. A. Sutheimer, and I. Sunshine, *Clin. Chem.*, (*Winston-Salem, N.C.*), **22**, 789 (1976).
(6) W. A. Trinler, D. J. Reuland, and T. B. Hiatt, *J. Forensic Sci. Soc.*, **16**, 133 (1976).
(7) E. G. C. Clarke, Ed., "Isolation and Identification of Drugs", William Clowes and Sons, London, 1974, pp 192–193.
(8) N. C. Jain, T. C. Sneath, and R. D. Budd, *Clin. Chem.* (*Winston-Salem, N.C.*), **20**, 1460 (1974).
(9) H. Jupille, *Am. Lab.*, **8**, 85 (1976).
(10) F. Nachtmann, H. Spitzy, and R. W. Frei, *Anal. Chem.*, **48**, 1576 (1976).
(11) P. Lebish, B. S. Fenkle, and J. W. Brackett, Jr., *Clin. Chem.* (*Winston-Salem, N.C.*), **16**, 195 (1970).
(12) J. E. O'Brian, W. Zazulak, V. Abbey, and O. Hinsvard, *J. Chromatogr. Sci.*, **10**, 336 (1972).

RECEIVED for review December 16, 1976. Accepted March 3, 1977.

Analysis of Acidic Monoamine Metabolites by Gas Chromatography–Mass Spectrometry

Damodar D. Godse,* Jerry J. Warsh, and Harvey C. Stancer

Section on Neurochemistry, Clarke Institute of Psychiatry, University of Toronto, 250 College Street, Toronto, Ontario, M5T 1R8 Canada

Derivatization of vanillylmandelic acid and 5-hydroxyindoleacetic acid using fluorinated and nonfluorinated alcohols and pentafluoropropionic anhydride produced a number of products. The structures of these products were determined by gas chromatography–mass spectrometry. Reaction conditions are described to substantially reduce the formation of undesirable products, thus making the procedures more suitable for the quantitative determination of these acidic metabolites in biological samples.

Gas chromatography–mass spectrometry (GC-MS) has recently been applied to the quantitative determination of aromatic monoamines and their metabolites in biological samples (*1–3*). Volatile monoamine derivatives for GC-MS analysis have been prepared through acylation with halogenated anhydrides such as trifluoroacetic, pentafluoropropionic, and heptafluorobutyric anhydride (*4*). For acidic monoamine metabolites, prior methylation of the carboxyl moiety with ethereal diazomethane followed by acylation with pentafluoropropionic or heptafluorobutyric anhydride has provided suitable volatile derivatives for analysis (*3, 5*). However, esterification of these metabolites using the above reagents may result in multiple products depending on the number of potential molecular sites of esterification and the reaction conditions employed (*6, 7*). These multiple products may give reduced sensitivity and also may result in loss of specificity in the case of selective ion monitoring GC-MS or gas–chromatography (GC) with electron capture detection.

Recently, less cumbersome procedures for determination of acidic monoamine metabolites have been reported (*8–10*). These methods employ halogenated alcohols such as hexafluoroisopropanol or pentafluoro-*n*-propanol for carboxyl esterification. Mass spectral verification of the derivatives formed has been reported (*9*) but little information was provided about potential by-products or products of incomplete reactions. In this paper we report a systematic investigation of the multiple esterification products of 5-hydroxyindoleacetic acid and vanillylmandelic acid using the principle of esterification described by Watson et al. (*9*) and describe alternative reaction conditions to obtain single derivatives by this chemical approach.

EXPERIMENTAL

5-Hydroxyindoleacetic acid (5-HIAA) and vanillylmandelic acid (VMA) were obtained from Sigma Chemical Co., St. Louis, Mo. Pentafluoropropionic anhydride, pentafluoro-*n*-propanol and trifluoroethanol were obtained from PCR Chemical Co., Gainesville, Fla. The latter reagents were redistilled prior to their use. For determination of mass spectra, 5 μg of each compound in 0.01 N HCl was evaporated to dryness under N_2 in a 1.0-mL reaction vial at room temperature. 5-HIAA and VMA were then derivatized with a mixture of pentafluoropropionic anhydride and pentafluoro-*n*-propanol (4:1) by the procedure of Watson et al. (*9*). The final reaction was terminated by evaporation of the reagents under N_2 and the products were reconstituted in 100 μL of redistilled dry ethyl acetate. Two-μL aliquots were injected for GC-MS analysis. For selective ion monitoring, 50 ng of each compound was treated as above and 2-μL aliquots were analyzed. Esterification of VMA was also examined using a mixture of pentafluoropropionic anhydride and trifluoroethanol in the same ratio as above.

Samples were analyzed using a Finnigan 3200 GC-MS system equipped with a programmable multiple ion monitor (Promim). Chromatographic separation was performed on a 1.5 m × 2 mm i.d. glass column, packed with 3% OV-17 on Gas Chrom Q, 100-120 mesh (Applied Science Laboratories, State College, Pa.). The injector port was maintained at 230 °C and the carrier gas (helium) flow rate was 20 mL/min. The separation of the products of VMA treated with pentafluoropropionic anhydride and pentafluoro-*n*-propanol was achieved at an oven temperature of 115 °C; for the products of VMA reacted with pentafluoropropionic anhydride and trifluoroethanol, an oven temperature of 130 °C was used. The products obtained following reaction of 5-HIAA with pentafluoropropionic anhydride and pentafluoro-*n*-propanol were separated by temperature programming from 145 to 200 °C at a rate of 10 °C/min after running isothermally at 145 °C for 60 s. Mass spectrometer conditions were as follows: separator temperature 230 °C; ion source temperature 70 °C; electron energy 70 eV; and emission current 1 mA. The solvent vehicle was vented through a diverter valve that was maintained open for 30 s following injection of the sample.

RESULTS

Using the reaction conditions described by Watson et al. (*9*) two distinct 5-HIAA esterification products were obtained. The first product was the pentafluoropropyl ester of 1,5-