degree of activity. The significance of further substitution in this comparative study is realized since some 2-monoalkyl and 2-hydroxyphenyl derivatives of the active 5-bromo-5-nitro-1,3-dioxane showed increased efficacy. These compounds offer some degree of selectivity to satisfy the multitude of antimicrobial requirements set forth for cosmetic and topical pharmaceutical products.

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GLC and NMR Analysis of Isomeric Impurities in the New Anti-Inflammatory Agent Benoxaprofen

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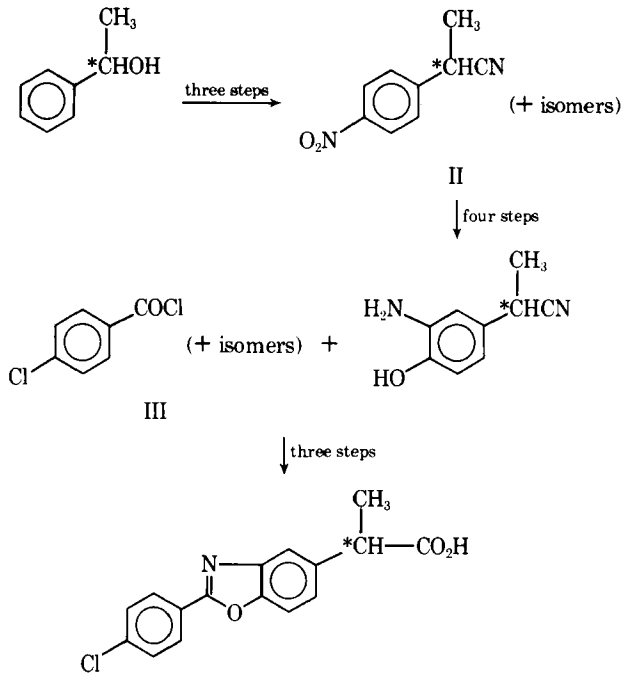
Abstract □ GLC and NMR methods are described for the determination of four possible isomeric impurities in the novel anti-inflammatory agent benoxaprofen. The 2- and 3-chlorophenyl isomers were determined by GLC after alkaline hydrolysis and subsequent methylation. A rapid NMR procedure, using the lanthanide shift reagent tris- (1,1,1,2,2,3,3-heptafluoro- 7,7-dimethyl- 4,6-octanedionato)-europium, was developed for the 6- and 7-(α-methylacetic acid) isomers. Similar methodology, with tris-(3-heptafluorobutyryl-*d*-camphorato)europium, enabled the determination of the enantiomer ratio for benoxaprofen. For the positional isomers, the limits of detection were 0.05% by GLC and 0.2% by NMR.

Keyphrases □ Benoxaprofen—with four isomeric impurities, GLC and NMR analyses □ GLC—analysis, benoxaprofen and isomeric impurities □ NMR—analysis, benoxaprofen and isomeric impurities □ Anti-inflammatory agents—benoxaprofen and four isomeric impurities, GLC and NMR analyses

Recently, the syntheses and anti-inflammatory activity of a number of 2-aryl-5-benzoxazoleacetic acids were described (1). The most active member of the series, 2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid (benoxaprofen, I), is several times more potent than phenylbutazone in the rat paw edema test and is currently under clinical evaluation.

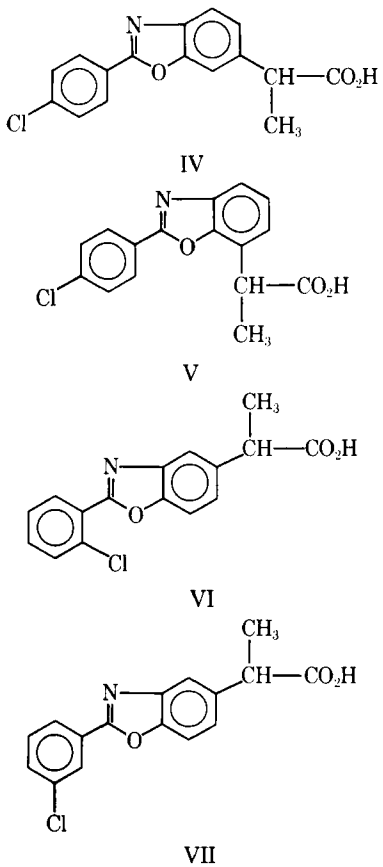
The material for toxicology and clinical requirements was prepared in a 10-step synthesis (Scheme I).

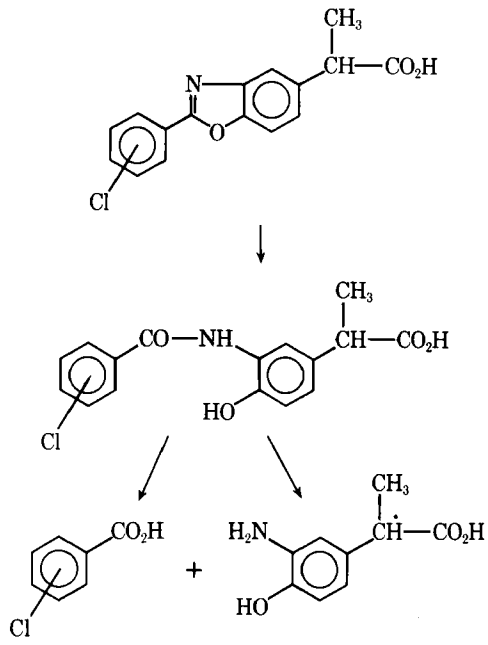
Isomeric impurities, which can arise at Steps 3 and



Scheme I— Asymmetric center*

8, might carry through to the final product and be very difficult to remove. Therefore, the isomer levels in II and III were carefully controlled. This paper describes the analytical procedures developed to estimate these four possible isomeric impurities, the 6- and 7-(α-methylacetic acid) and 2- and 3-chlorophenyl isomers (IV–VII), in the final product.





Scheme II

Attempts to achieve a separation of low concentrations of all four isomers of I by GLC using standard packed columns proved unsuccessful. These compounds, derivatized as their methyl esters, were not resolved on a number of different columns. However, hydrolytic ring fragmentation of I, VI, and VII yielded the corresponding chlorobenzoic acids (Scheme II), which were separated and determined as their methyl esters.

In the absence of a suitable GLC method for determining IV and V, a rapid NMR procedure was developed using the fluorinated europium shift reagent, tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium (VIII) (2). An optically active analog of this reagent also separates the NMR signals for the optical isomers of I, allowing their determination.

EXPERIMENTAL

Determination of Low Concentrations of 2- and 3-Chlorophenyl Isomers of I—*Reagents*—Compounds IV–VII were of at least 95% purity[1].

Acetone[2] and ether[3] were analytical reagent grade. Sodium hydroxide[2], hydrochloric acid[3], 4-chloroacetophenone[2], 4-chlorobenzoic acid[4], 3-chlorobenzoic acid[2], and 2-chlorobenzoic acid[2] were used as purchased. While the 4- and 2-chlorobenzoic acid samples showed <0.1% of the other isomers, the 3-chlorobenzoic acid contained 2.4% of the 4-isomer and 1.7% of the 2-isomer; an overall purity of 96% 3-isomer was assumed.

The diazomethane solution was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[5] according to the manufacturer's recommended procedure. All operations with this reagent were carried out in a fume cupboard.

GLC—For the GLC determinations, a gas chromatograph[6] equipped with a hydrogen flame-ionization detector (hydrogen 30 ml/min and air 300 ml/min) was used. The glass column [1.8 m × 0.6

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[2] British Drug House Chemicals Ltd.
[3] May & Baker Ltd.
[4] Koch-Light Laboratories Ltd.
[5] Aldrich Chemical Co.
[6] Pye Unicam, model GCV.

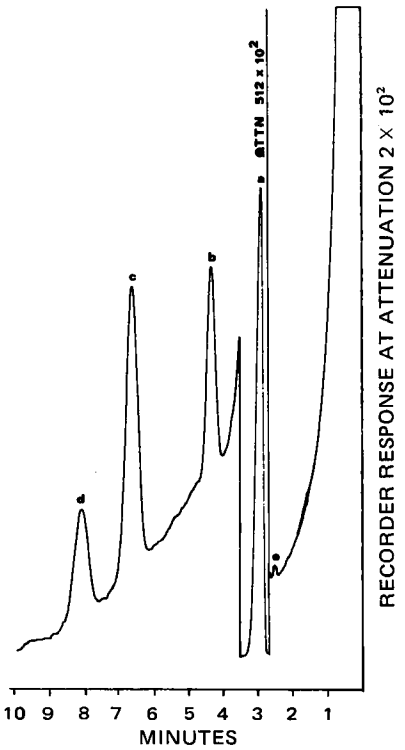

Figure 1—*Gas chromatogram from a sample containing 0.5% of VI and VII in I. Key: a, main component, methyl 4-chlorobenzoate; b, methyl 3-chlorobenzoate; c, internal standard, 4-chloroacetophenone; d, methyl 2-chlorobenzoate; and e, peak present in blank. The conditions were: 5% IX–4% diisooctyl phthalate on 80–100-mesh Gas Chrom Q, nitrogen flow rate of 30 ml/min, and column temperature of 180°.*

cm (3 ft × 0.25 in.) o.d.] was packed with 5% dimethyldioctadecylammonium bentonite[7] (IX) and 4% diisooctyl phthalate[2] on 80–100-mesh Gas Chrom Q[8]. The stationary phases were simultaneously coated on the solid support by the slurry technique. Nitrogen was used as carrier gas at 30 ml/min. The temperatures were as follows: column, 180°; injection port, 190°; and detector, 230°. The samples were injected on column.

Under these conditions, the retention times were: methyl 4-chlorobenzoate, 3.1 min; methyl 3-chlorobenzoate, 4.5 min; 4-chloroacetophenone, 6.8 min; and methyl 2-chlorobenzoate, 8.1 min.

A chromatogram obtained from a sample containing 0.5% of VI and VII in I is shown in Fig. 1. Quantitation was done using peak height ratios.

Calibration Graphs—The following reference compound solutions were prepared. The internal standard solution was 4-chloroacetophenone, 0.03 mg/ml, dissolved in acetone.

For stock solution of 2- and 3-chlorobenzoic acids, 50 mg of each acid was dissolved in ether and diluted to 50.0 ml. Then 1.0 ml of this solution was pipetted into a 50-ml volumetric flask and diluted to volume with ether.

For the working solutions, aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 ml of the stock solution were pipetted into 10-ml glass vials, and the solvent was evaporated to dryness in a fume cupboard under a stream of nitrogen. Several drops of diazomethane solution were carefully added, and the vial was lightly capped and left for about 10 min. If the solution was colorless, more diazomethane was added, and the procedure was repeated. If the solution was yellow, excess diazomethane was present, and the solution was then evaporated to dryness under a fume hood.

The residues were each dissolved in 1.0 ml of the internal standard solution. These solutions then contained approximately 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml of both 2- and 3-chlorobenzoic acid methyl esters. A 5-μl sample of each solution was injected into the chromatograph, and a peak height of each component was measured. Calibration graphs were obtained by plotting the peak height ratios of each methyl ester to the internal standard as a function of the concentration of the methyl ester. These were linear and passed through the origin.

Hydrolysis—About 50 mg of accurately weighed sample was placed in a 100-ml round-bottom flask, and 10 ml of 2 *N* sodium hydroxide was added. The solution was heated under reflux for 4 hr, cooled, and transferred to a 50-ml separator with water. Hydrochloric acid (5 ml of 5 *N*) was added, and the solution was extracted with two 10-ml aliquots of ether.

The ether extracts were combined, transferred to a 25-ml volumetric flask, and diluted to volume with ether. A 5.0-ml aliquot of this solution was pipetted into a 10-ml glass vial and evaporated to dryness under a stream of nitrogen in a fume cupboard. The residue was dissolved in excess diazomethane, lightly capped, and left for about 10 min. The excess diazomethane was evaporated to dryness, and the residue was dissolved in 1.0 ml of internal standard solution.

Then 5 μl of the solution was injected into the gas chromatograph. The peak heights of the methyl esters of 3- and 2-chlorobenzoic acids and of the internal standard were measured, and the appropriate ratios were calculated. Their concentrations were then determined from the calibration graph, and the concentrations of VI and VII present in the sample were calculated.

Recovery Experiments—Samples of about 50 mg of I, VI, and VII were weighed into 100-ml round-bottom flasks, 10 ml of 2 *N* sodium hydroxide was added, and the solutions were heated under reflux for 4 hr. These solutions were then cooled, acidified, and extracted as described for the hydrolysis procedure. A 5.0-ml aliquot of each ether extract was pipetted into 100-ml volumetric flasks and diluted to volume with ether. One-milliliter aliquots of each solution were pipetted into 10-ml glass vials and methylated as in the hydrolysis procedure.

Recovery calculations on VI and VII were determined using the calibration graphs, while those on I were obtained by plotting a similar calibration graph using 4-chlorobenzoic acid. Synthetic mixtures of VI and VII at the 0.1, 0.2, 0.5, and 1.0% levels in I were prepared and taken through the hydrolysis procedure. The recovery figures are shown in Table I.

Determination of VI and VII in I—Accurate weighings of about 50 mg of sample were taken through the hydrolysis procedure, and concentrations of VI and VII were determined by means of the standard calibration curves as follows:

$$\text{percentage of VII} = \frac{(\text{mg of methyl 3-chlorobenzoate produced}) \times 301.7 \times 100 \times 5}{(\text{sample weight, mg}) \times 170.5} \quad \text{(Eq. 1)}$$

$$\text{percentage of VI} = \frac{(\text{mg of methyl 2-chlorobenzoate produced}) \times 301.7 \times 5 \times 100 \times 1.22}{(\text{sample weight, mg}) \times 170.5} \quad \text{(Eq. 2)}$$

where 1.22 is a correction factor for 82% recovery.

Determination of Low Concentrations of 6- and 7-(α-Methylacetic Acid) Isomers of I—*Instruments and Reagents*—NMR spectra were obtained as 250 accumulations on a 90-MHz Fourier transform NMR spectrometer[9] or on a 60-MHz continuous-wave spectrometer[10]. Deuterochloroform[11], containing 1% (v/v) tetramethylsilane[11], was used as the solvent. Spectral scans (δ 0–13) were referred to the singlet due to tetramethylsilane set on the chart at δ = 0.00.

Deuterochloroform (99.8%) was dried over molecular sieve 4A[2] before use. Tetramethylsilane, VIII[11], and tris-(3-heptafluorobutyryl-*d*-camphorato)europium[12] were used as purchased.

Determination of IV and V in I—A 10.0-mg sample of the appropriate acid was treated with 1 ml of a 0.25 *M* ethereal diazomethane solution for 5 min at room temperature. The unreacted reagent and solvent were evaporated in a stream of nitrogen, and the resulting methyl ester was dissolved in 0.35 ml of deuterochloroform. Com-

[7] Bentone 34, Phase Separations Ltd.
[8] Applied Science Laboratories.
[9] Bruker WH90.
[10] Varian A-60A.
[11] Ryvan Chemical Co. Ltd.
[12] Willow Brook Laboratories Ltd.

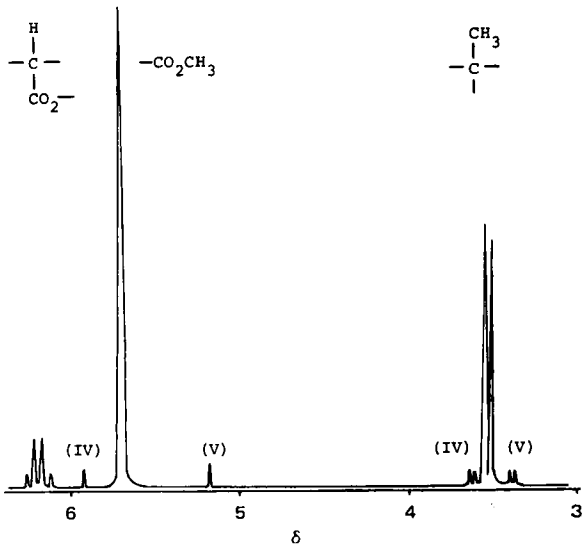

Figure 2—*Part spectrum of I (containing ~3% added IV and V) treated with 0.38 equivalent of VIII. Peaks due to the methyl groups of IV and V are indicated in parentheses.*

pound VIII (13 mg, 0.38 equivalent) was added, and the NMR spectrum was examined in the δ 2.5–6.5 region (Fig. 2). The peaks occurring in the δ 5–6 region in the lanthanide-shifted spectrum were due to the methyl ester protons of I or its 6- or 7-isomer (I = δ 5.69, IV = δ 5.90, and V = δ 5.14). Quantitation of the isomeric impurities followed from the duplicate measurement of the ratio of peak heights for the relevant methyl ester protons. Ordinate expansion by a factor of 8 was used to amplify the small peaks due to IV or V.

Determination of Enantiomers of I—Compound I (10.0 mg) was treated with diazomethane as already described, and the resulting methyl ester was dissolved in 0.35 ml of deuterochloroform. To this solution, 11.0 mg (0.28 equivalent) of tris-(3-heptafluorobutyryl-*d*-camphorato)europium was added, and the NMR spectrum was recorded in the δ 2–6 region (Fig. 3). The enantiomer ratio was determined by measuring the relative heights of the low field peaks for the side-chain methyl doublets (at δ 2.55 and 2.61).

RESULTS AND DISCUSSION

Column—Raley and Kaufman (3) reported the separation of the isomeric monochlorostyrenes using an organo clay[7] (IX) modified with

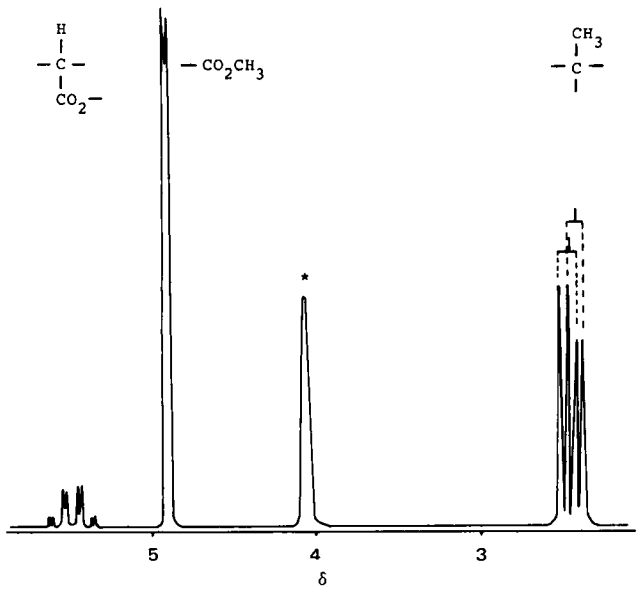

Figure 3—*Part spectrum of I treated with 0.28 equivalent of tris-(3-heptafluorobutyryl-*d*-camphorato)europium. Starred peak is due to europium reagent.*

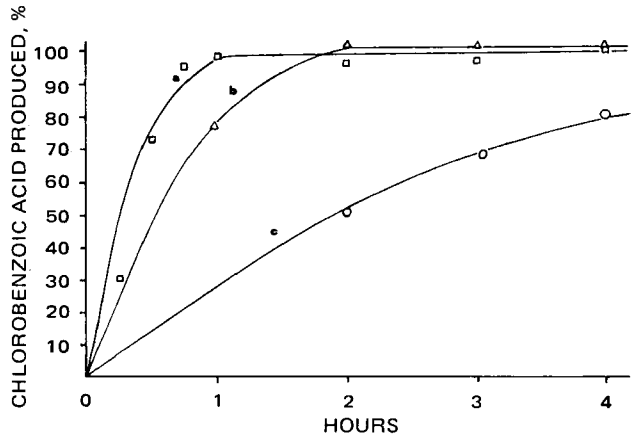

Figure 4—*Amounts of chlorobenzoic acids produced from I, VI, and VII with varying hydrolysis times. Key: a, 4-chlorobenzoic acid produced by hydrolysis of I; b, 3-chlorobenzoic acid produced by hydrolysis of VII; and c, 2-chlorobenzoic acid produced by hydrolysis of VI.*

other stationary phases. Compound IX modified with diisooctyl phthalate also has been used to separate the isomers of benzoyl chloride. Although the temperature limit of IX is too low to be of use for separating VI and VII, the methyl esters of the chlorobenzoic acids produced after hydrolysis can be separated with this phase. The most suitable column was a 1.8-m × 0.6-cm (3-ft × 0.25-in.) o.d. glass column packed with 5% IX–4% diisooctyl phthalate on 80–100-mesh Gas Chrom Q. It gave good resolution of the isomeric methyl chlorobenzoates with good peak shapes and minimum retention times (Fig. 1).

Hydrolysis Conditions—Preliminary studies showed that alkaline hydrolysis proceeded more rapidly than acid hydrolysis. Figure 4 shows the effect of varying hydrolysis time on the amount of chlorobenzoic acids produced from I, VI, and VII. Although hydrolysis of the main component, I, was complete in about 1 hr and hydrolysis of VII was complete in about 2 hr, VI required a longer time. And even after 4 hr, only 82% of the 2-chlorobenzoic acid had been produced. Consequently, a hydrolysis time of 4 hr was chosen, and the figures obtained from 2-chlorobenzoic acid were corrected accordingly.

Recovery Data—Table I shows the recovery figures obtained on samples of I, VI, VII, and several mixtures: approximately 100% for I and VII and 80% for VI. Recoveries were significantly lower in the 0.1% mixture, as the limit of detection was approached.

Analytical Performance—With the described method, VI and VII can be determined in the 0.1–1.0% range in I with a precision of ±10%. Higher levels can be easily determined by appropriate reduction of the size of aliquot taken in the hydrolysis procedure. Blank determinations show no interference. The limit of detection is about 0.05% of either isomer in a 50-mg sample.

NMR Conditions—NMR spectroscopy, with the aid of a lanthanide shift reagent (2, 4), provides a facile separation of signals from the 5-, 6-, and 7-isomers, enabling their quantitation. A sample of I, containing added amounts of IV and V, showed no separation of methyl ester peaks in the 90-MHz NMR spectrum after methylation. However, treatment with 0.38 equivalent of VIII caused the methyl ester signal of all three components to separate clearly (Fig. 2).

Further addition of shift reagent was not advantageous because of overlap of the methyl ester resonance of IV with the methine resonance of I. As shown in Fig. 2, the doublet signals for the methyl groups in the α-methylacetic acid side chain were separately resolved in the presence of VIII. The smaller separations and split nature of these signals make them an unattractive alternative to the procedure using the singlet of the methyl ester group.

Quantitation and Detection Limits—Quantitation was undertaken using peak height measurements rather than integrated areas to avoid problems of integral drift at low signal levels. Results obtained on standard mixtures are given in Table II. For the solution containing 0.31% of IV, the signal height for the methyl ester group of IV was four times the average noise excursion. Hence, the detection limit for IV and V is about 0.2%. On the continuous-wave instrument, the practical limit of detection was an order of magnitude poorer (3–4%), demonstrating the sensitivity advantages of the Fourier technique.

Table I—Recovery Data Obtained by Hydrolyzing Standard Mixtures of I, VI, and VII[a]

Sample	I			VI			VII		
	Milligrams Present	Milligrams Found	Average Recovery, %	Milligrams Present	Milligrams Found	Average Recovery, %	Milligrams Present	Milligrams Found	Average Recovery, %
Pure compound	51.4	49.5 ± 1.9	96	48.5	39.3 ± 0.3	81	49.3	48.0 ± 0.3	97
1% of VI and VII in reaction product	52.4	56.6 ± 0.6	108	0.564	0.467 ± 0.03	83	0.495	0.535 ± 0.06	108
0.5% of VI and VII in reaction product	50.7	55.3 ± 0.3	109	0.282	0.241 ± 0.013	85	0.248	0.281 ± 0.011	113
0.2% of VI and VII in reaction product	54.3	52.4 ± 4.9	97	0.113	0.092	81	0.099	0.093 ± 0.005	94
0.1% of VI and VII in reaction product	53.0	45.7 ± 2.1	86	0.056	0.040 ± 0.001	70	0.050	0.038 ± 0.001	76

[a] Individual figures quoted are usually the mean of two determinations.

Table II—Analyses of IV and V in I by NMR

Component	Added, % (w/w)	Found, % (w/w)
IV	2.9	2.2, 2.8
IV	0.31	0.38, 0.32
V	3.1	3.0, 3.5
	3.0	2.9, 3.5

Determination of Enantiomeric Ratio for I—Optically active lanthanide shift reagents have been applied successfully in the resolution of NMR signals of enantiomers (5). Addition of 0.28 equivalent of tris-(3-heptafluorobutyryl-*d*-camphorato)europium caused a useful δ 0.06 separation of the side-chain methyl doublets in the two enantiomers of the methyl ester of I (Fig. 3).

Addition of further shift reagent caused first an overlap of the middle peaks as the methyl doublets crossed and then excessive line broadening. It was possible to estimate the enantiomeric ratio to an accuracy of approximately ±3%. As expected from the mode of synthesis, the compound was racemic. This rapid procedure may prove valuable in the evaluation of the enantiomeric content of other anti-inflammatory acids, *e.g.*, for raw materials or in metabolic studies (6, 7).

CONCLUSION

A number of samples of I synthesized from different batches of intermediates, II and III, with isomeric impurities controlled to less than 0.5% in each, were examined for traces of IV–VII. None of the samples showed any detectable quantities of these compounds, indicating that adequate control of intermediate purity had been achieved and that impurities were not concentrated during the synthesis.

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