NMR Assay of Diastereoisomers of 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one Using Deuterated Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III Shift Reagent

PHILIP REISBERG[x], **IAN A. BRENNER, and JEROME I. BODIN**

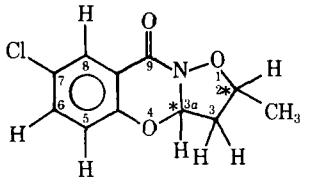
Abstract □ A simple, rapid, and quantitative NMR method was developed for the determination of the ratio of the diastereoisomers present in 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one (I). Deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III shift reagent causes the doublet assigned to the protons of the 2-methyl group, which normally appear at about 1.5 ppm, to split into a pair of doublets and shifts them downfield to about 3 ppm. Each new doublet represents one racemic diastereoisomeric pair present in I. The ratio of the pairs was determined by NMR integration of the areas under the respective doublets by calculating their proportions relative to the total area under the doublets. When a series of prepared mixtures of the isolated pairs was analyzed and their ratios were determined, the results obtained were within 1% of their actual values.

Keyphrases □ 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-b)(1,3)benzoxazin-9-one diastereoisomers—NMR analysis, deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III shift reagent □ Shift reagents—deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III for NMR analysis of diastereoisomers of 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one □ Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)-europium III shift reagent—NMR analysis of 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one diastereoisomers □ NMR—analysis, 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one diastereoisomers

7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-b)(1,3)benzoxazin-9-one[1] (I) is a member of a new class of anti-inflammatory compounds first synthesized by Reisner and Ludwig (1, 2). It is of particular chemical interest since it is a member of a newly reported heterocyclic ring system. The presence of an isoxazole ring contiguous to a 1,3-benzoxazine ring is the unique feature of the system. Because of the presence, at positions 2 and 3*a* (starred), of two asymmetric carbon atoms, four isomers theoretically should occur, resulting in two pairs of diastereoisomers, each consisting of two enantiomers.

DISCUSSION

A quantitative method to determine the proportions of each diastereoisomeric pair present in I was required. Separation by column chromatography was feasible but proved to be long, arduous, and difficult to reproduce quantitatively. However, preparative separation by column chromatography was performed to isolate the two diastereoisomeric pairs, which were designated as the α- and β-forms of I. Expectedly, they had different properties, *e.g.*, melting range, R_f value by TLC, and NMR spectrum.



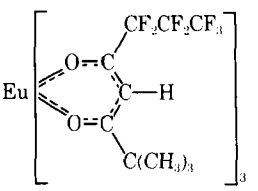
I

It was thought that the difference in their NMR spectra might be used for quantitative analysis. In 1966, van der Vlies *et al.* (3) reported the determination of the ratio of the diastereoisomeric pairs of cyclandelate by NMR spectroscopy. They found a difference in the positions of the peaks in the methyl proton region of the NMR spectrum, which enabled them to determine the ratio by measuring peak heights.

Compound I presented a somewhat different problem since the NMR spectra of its two diastereoisomeric pairs (Fig. 1) do not contain a suitable group for such use. Although there are distinct differences in the splitting patterns of the protons at 2, 3, and 3*a* in the isolated pairs, it is not possible to distinguish the two in combination. In Fig. 2 the spectrum of I appears almost the same as that of the α-form with some slight contamination by the β-form. There is a faint indication of two methyl proton absorption doublets, but these are too close to be of any value for quantitative purposes. A procedure for their separation was required if the NMR method was to be successful.

The use of paramagnetic complexing agents to produce large chemical shifts was reported by Hinckley (4), who observed large shifts in the NMR spectrum of cholesterol in the presence of the pyridine complex of tris(dipivaloylmethanato)europium III (II). Sanders and Williams (5) improved on this method by using the pyridine-free complex of II and increased the observed shifts by a factor of four. Many other lanthanide complexes were investigated, with the result that europium and praseodymium complexes were found to be most effective and to cause the least line broadening. Fortunately, these were complementary in that europium complexes produce a downfield shift, while praseodymium complexes produce an upfield shift. The magnitude of the shift depends on the relative molar proportions of the sample and lanthanide shift reagent used.

Initial work in this laboratory involved the use of the new complex, tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III (III), first reported by Rondeau and Sievers (6). This compound is more soluble and is a better complexing agent



III

[1] Previously referred to in the literature as W-2395.

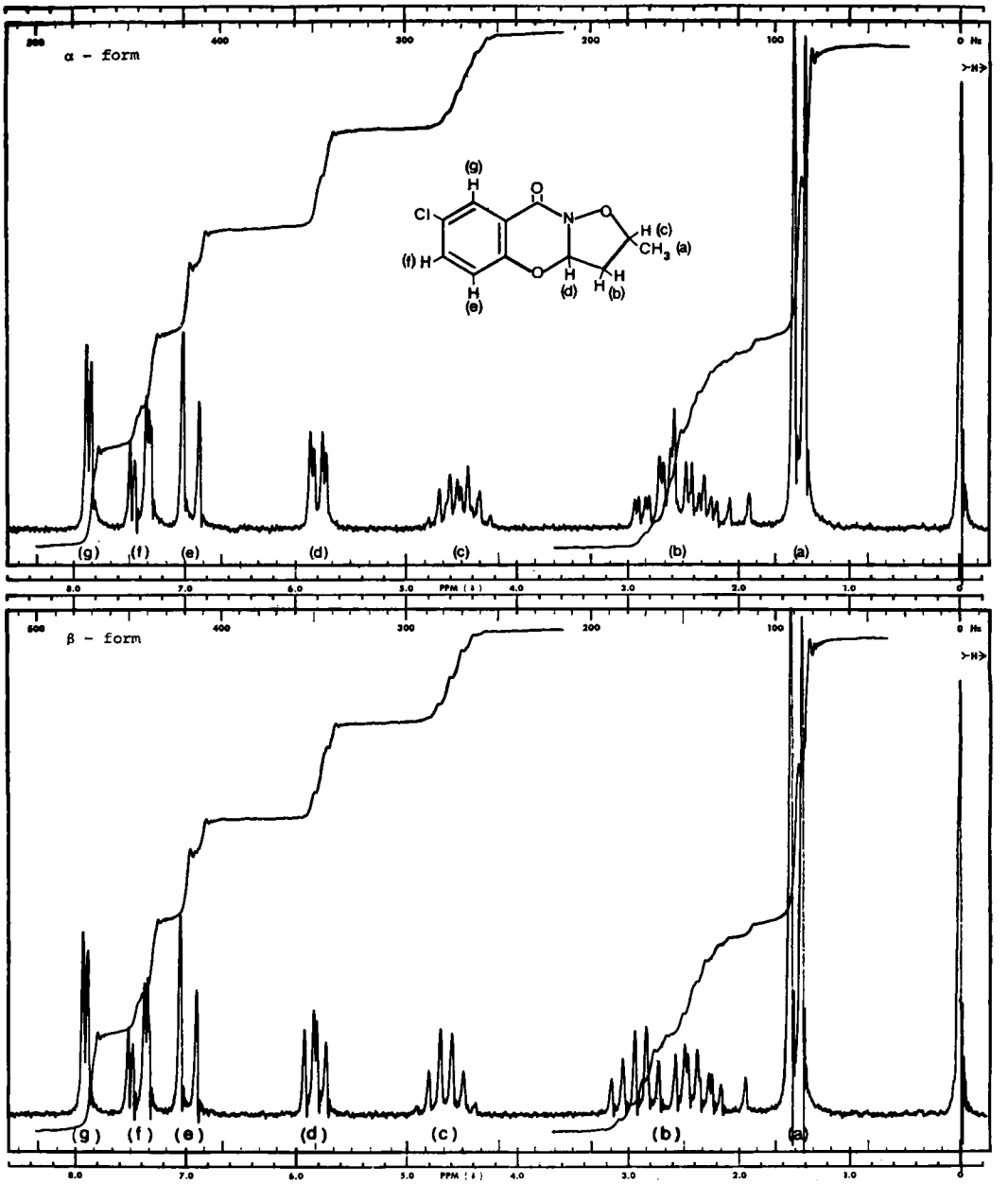


Figure 1—*NMR spectra of α- and β-forms of I in $CDCl_3$.*

than previous lanthanide shift reagents due to increased Lewis acid activity. A slight difficulty due to the absorption of the protons of the nine methyl groups in the 1.0–2.0-ppm region has recently been circumvented by the introduction of the deuterated III compound. The latter reagent, which absorbs only slightly in the 1.0–2.0-ppm region, was considered the reagent of choice.

EXPERIMENTAL

Apparatus—Spectra were obtained at 60 MHz using an analytical spectrophotometer[2]. A sweep time of 250 sec and a sweep width of 500 Hz were used, unless indicated otherwise; the δ-scale was used throughout. Tetramethylsilane (IV) (1% v/v) in deuterated chloroform[3] was used as the internal reference from which to measure chemical shifts.

Reagents and Chemicals—*Compound I*—The purity (sum of the α- and β-forms) was determined by dissolving an accurately weighed sample (about 500 mg) in 50 ml of 0.1 *N* NaOH, refluxing on a hot plate for 5 min, and back-titrating with 0.1 *N* H_2SO_4 using phenolphthalein TS as the indicator. Each milliliter of 0.1 *N* NaOH is equivalent to 23.97 mg of I.

α- and β-Forms—Compound I was separated into its two diastereoisomeric pairs (α and β) by absorption column chromatography on alumina[4] using ethyl acetate–ether (1:1) as the eluent. The presence of the two pairs in the eluate fractions was monitored by TLC. Samples of the eluate were spotted on silica gel plates[5], developed with ether–ethyl acetate (1:1), dried, and viewed under shortwave UV light. The initial fractions, which gave a single spot at R_f 0.48, were collected and evaporated to dryness. The residues were recrystallized from trichloroethylene and the product was assigned the designation α. Subsequent eluates, which gave a single spot on TLC plates at R_f 0.30, were also collected and evaporated to dryness. The product was recrystallized from methanol and given the designation β. Eluates giving more than one spot by TLC were rechromatographed until a fraction was obtained that gave only one spot.

[2] Varian T-60.
[3] Norell Chemical Co., Landing, N.J.
[4] Woelm neutral activity 1.
[5] F254, E. Merck.

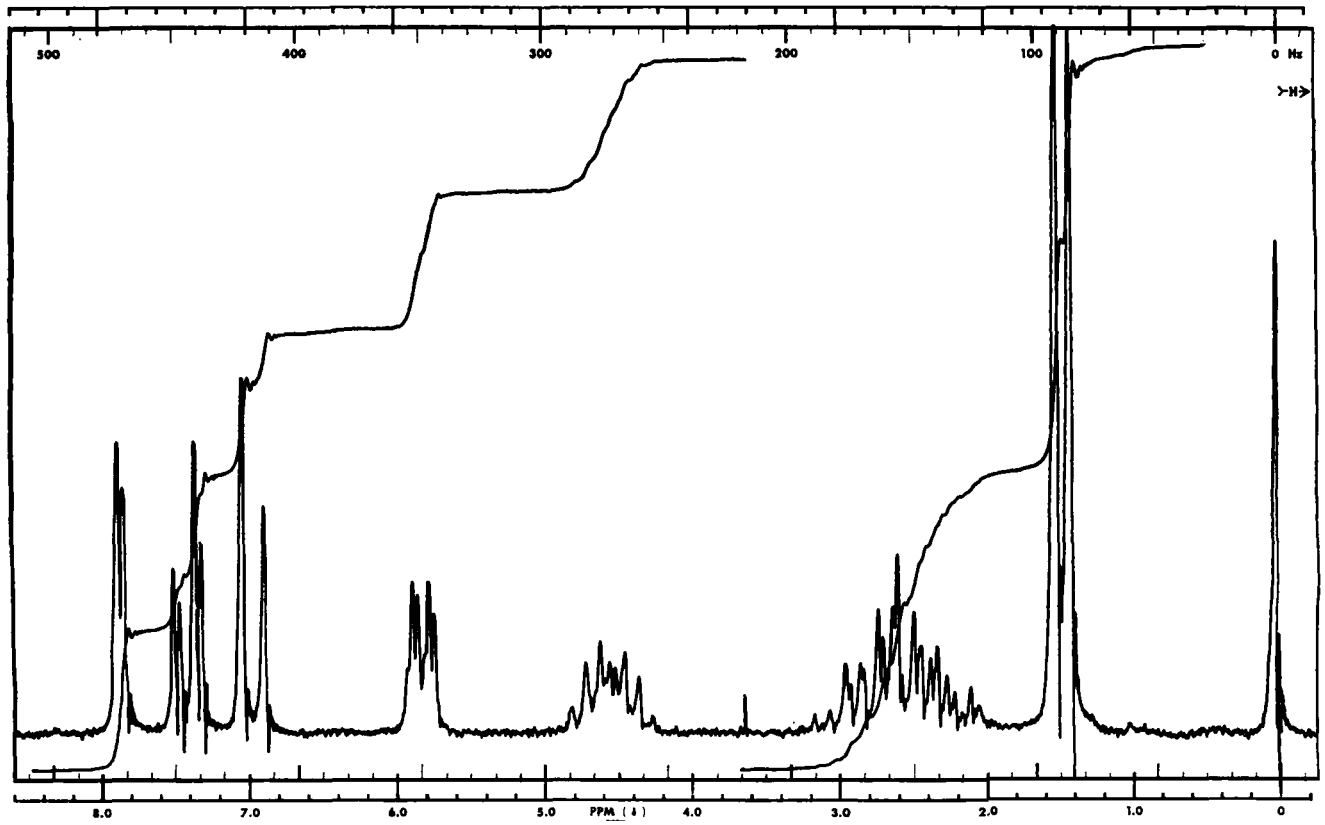


Figure 2—*NMR spectrum of I in $CDCl_3$.*

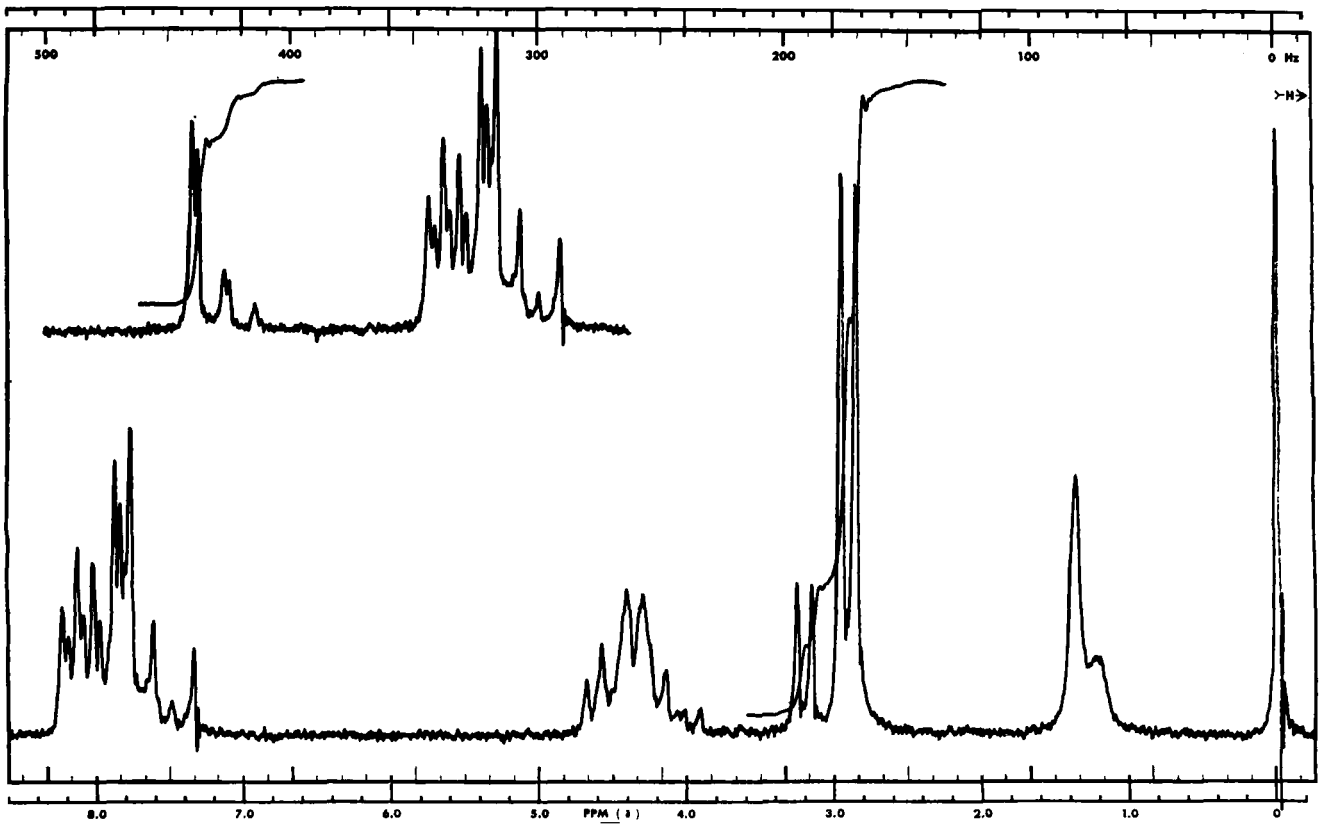
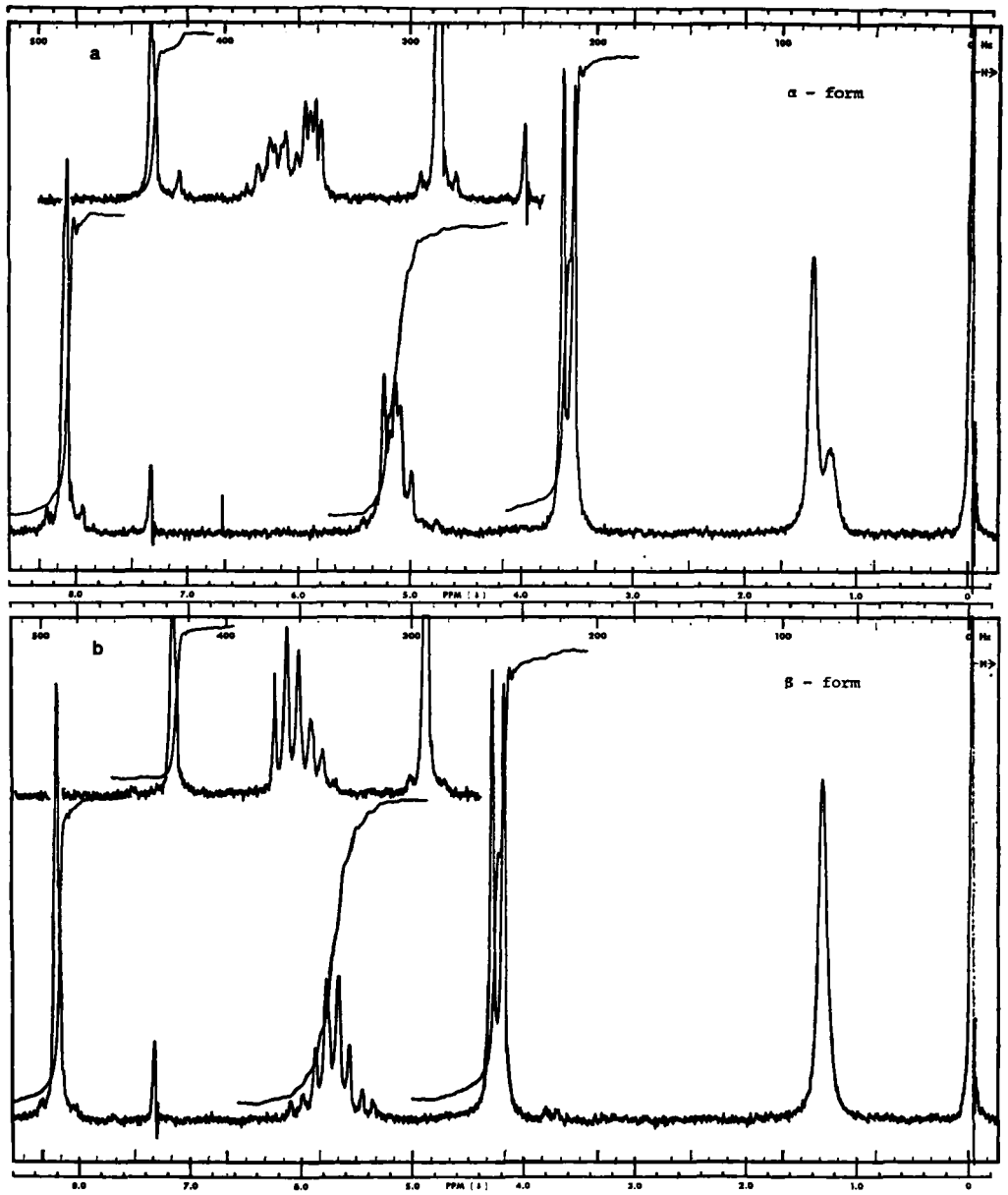


Figure 3—*NMR spectrum of I + deuterated III in $CDCl_3$.*



igure 4—(a) *NMR spectrum of 0.14 mmole α-form + 0.14 mmole deuterated III in $CDCl_3$.* (b) *NMR spectrum of 0.14 mmole form + 0.14 mmole deuterated III in $CDCl_3$.*

Lanthanide Shift Reagent—This was prepared by mixing 2105 g of deuterated III with 3.53 ml of deuterated chloroform, ntaining 1% of IV, and centrifuging the mixture to remove un-ssolved material. This concentration was found to be optimal.

Procedure—*Method A*—Solutions of 60 mg of I and the α- and forms in 0.45 ml of deuterated chloroform containing 1% of IV ere prepared separately in NMR tubes[6], and their NMR spectra ere obtained (Figs. 1 and 2).

Method B—About 33 mg (0.14 mmole) of I and the α- and β-rms were dissolved separately in 0.45 ml of lanthanide shift re-ent, and their NMR spectra were obtained (Figs. 3 and 4).

Method C—Prepared samples of I were made by accurately eighing the quantities shown in Table I, dissolving them in 0.45 l of the lanthanide shift reagent, and obtaining their NMR spec-a. The spectra were expanded to a 250- and 100-Hz sweep width d integrated five times through the region of interest (2.5–3.0 pm). The mean of the integrated values was used for quantitative lculations (Figs. 5 and 6).

Calculation—The percentage composition of each diastereoisomeric pair was determined as follows:

$$\% \alpha = \frac{A_1 \times 100}{A_1 + A_2} \quad \text{(Eq. 1)}$$

$$\% \beta = \frac{A_2 \times 100}{A_1 + A_2} \quad \text{(Eq. 2)}$$

where A_1 = area of upfield doublet (at about 30 ppm) and A_2 = area of downfield doublet (at about 3.3 ppm).

RESULTS

The NMR spectra of I and the α- and β-forms are shown in Figs. 1 and 2. The NMR signal for the protons of the methyl group at 1.5 ppm is split into a doublet by the proton on the adjacent carbon atom. The chemical shift for these protons is essentially the same for all three compounds.

Upon addition of the deuterated III shift reagent to I, a large downfield shift in the NMR spectrum was observed for all peaks due to the protons of ring 3 (Fig. 3). However, unexpectedly, the signal for the protons of the methyl doublet was shifted unequally

[6] No. 507, Wilmad Glass Co., Buena, N.J.

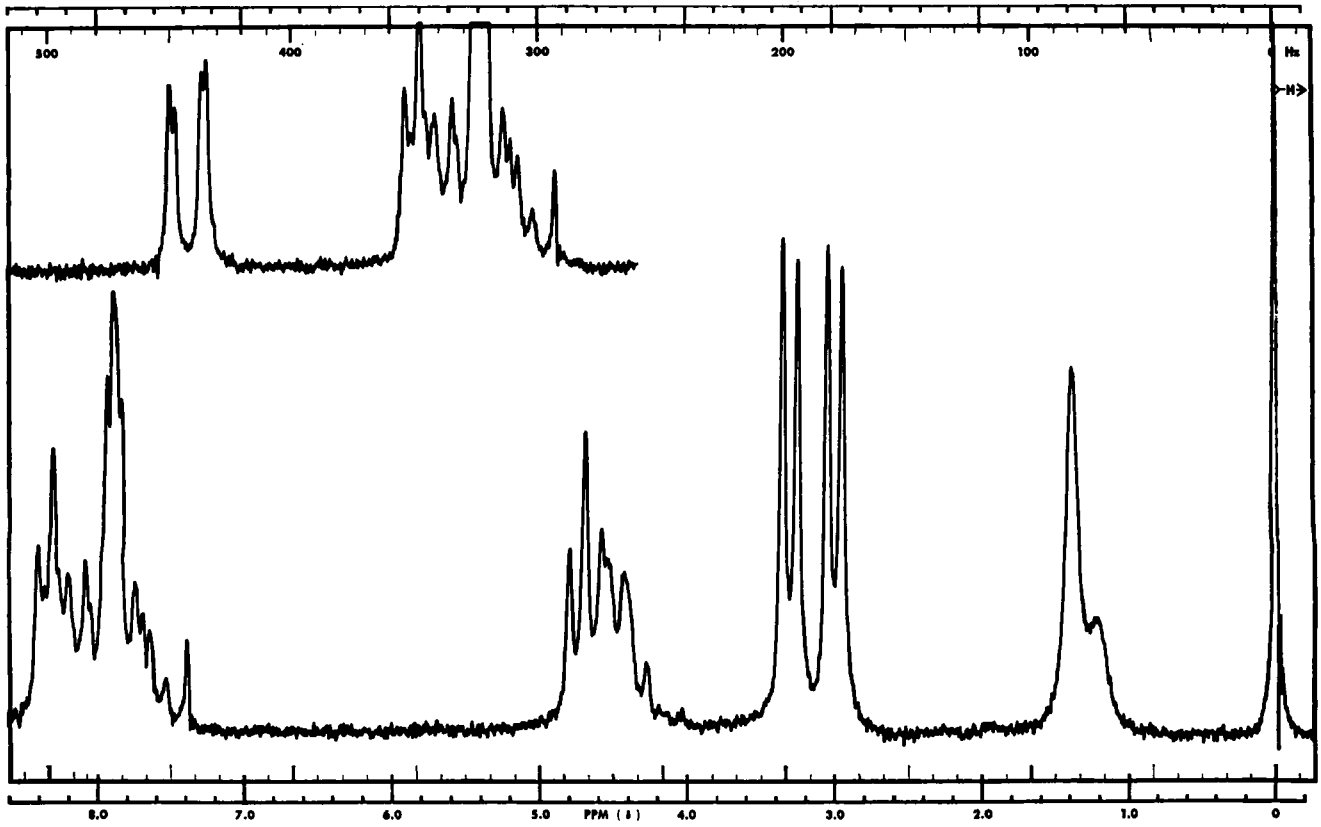


Figure 5—*NMR spectrum of prepared mixture of I (1:1 α-form–β-form) + deuterated III in $CDCl_3$.*

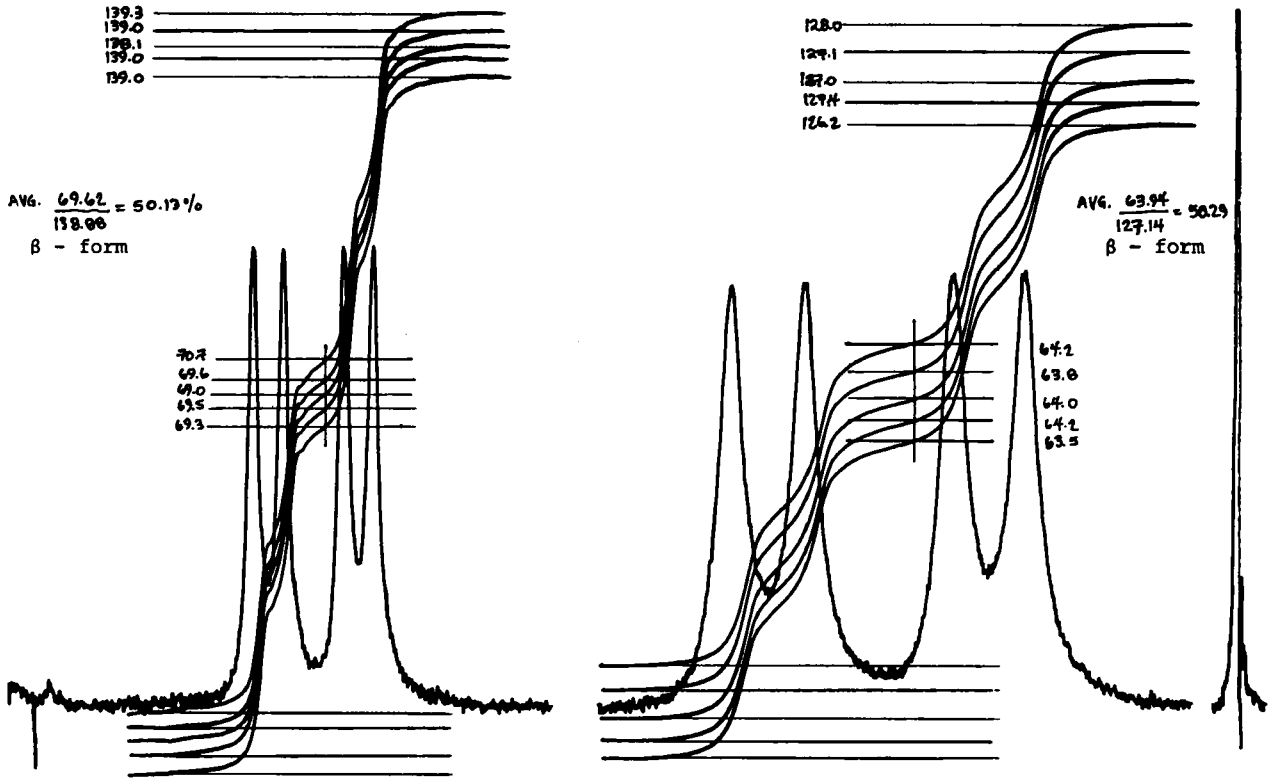


Figure 6—*The 250-Hz (left) and 100-Hz (right) sweep width expansion of methyl absorbances in Fig. 5.*

and was separated into two distinct doublets at about 3 ppm. Each doublet represents the methyl group present in either the α- or β-form. The NMR signals for the other protons in ring 3 were not separated but were still overlapping; one group had been shifted completely into the aromatic region.

This downfield shift probably results from the coordination of the deuterated III with a pair of electrons on oxygen or nitrogen of the isoxazole ring. The difference in the NMR signal of the methyl

Table I—Analysis of Prepared Samples of Compound I by NMR

Sample	α-Form, mg	β-Form, mg	Percent α	Percent α Found	Percent Recovered
1	57.50	3.20	94.73	94.88	100.17
2	54.12	6.31	89.56	89.78	100.25
3	42.66	19.00	69.19	69.80	100.88
4	30.38	31.20	49.33	49.79	100.93
5	28.62	33.52	46.06	46.08	100.04
6	23.80	36.38	39.55	39.86	100.78
		Mean	100.51		
		SD	0.40		

protons is probably due to the difference in the geometry of the two diastereoisomeric pairs and the manner in which the lanthanide shift reagent contacts them. This produces a greater effect in one case than in the other.

Positive identification of each methyl doublet was determined (Fig. 4). Under the same conditions, the NMR signal for the protons of the methyl doublet of the β-form was shifted further downfield than was that for the α-form. This finding confirms what was apparent from the examination of Fig. 3 where the downfield doublet is much smaller than the one upfield.

Table I reports the results of NMR analyses of prepared samples of I. Figures 5 and 6 show the NMR spectra of one of these mixtures, their expansion, and their integration. The recovery studies of these samples resulted in a mean of 100.51% with a standard deviation of ±0.40. There appears to be a constant positive bias which could result from the slight contamination of the β-form with the α-form. This is quite reasonable because α was easily obtained in pure form while β was separated from α only with great difficulty.

Two commercial size batches of I were made by the same process and gave ratios of 83.3% α to 16.7% of β and 82.9% α to 17.2% of β, respectively. The overall purity of these batches was 99.7% as determined by the method given in the *Experimental* section.

The accuracy and simplicity of this NMR method make it very useful and rapid for determining the relative proportions of the diastereoisomeric pairs of I.

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Progesterone Injection Assay by Liquid Chromatography

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Abstract □ An assay was developed for the progesterone content of injections composed of refined, fixed oils as the primary solvent. Reversed-phase, high-pressure liquid chromatography separates the progesterone from all other known ingredients following initial extraction into aqueous ethanol. Methyltestosterone was found to be a desirable internal standard, and peaks were measured photometrically. Twelve injection samples and six standards can be completed in 1 day. A system suitability test was defined. Evidence is presented for the recovery, specificity, accuracy, and precision of the method.

Keyphrases □ Progesterone injection—analysis by high-pressure liquid chromatography □ Steroids—analysis of progesterone injection by high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, progesterone injection

The official assay for progesterone in oil is a gravimetric determination of the double 2,4-dinitrophenylhydrazone adduct (1). This assay is unspecific, measuring any ketosteroid content, and serious problems have been observed with the quality of the precipitating reagent and with formation of the precipitate. A new assay with notably improved specificity was required in light of apparent problems with marketed items.

Analytical approaches to drugs in oil-based pharmaceuticals (2) and the pharmaceutical aspects of high-pressure liquid chromatography (HPLC) (3) have been reviewed. Specific, but cumbersome, progesterone determinations have been advanced including an IR partition column assay (4) and an extraction method followed by GLC (5). The purposes of this study were to develop and to define a specific and simple assay that utilizes the advantages offered by HPLC in the analysis of steroids.

EXPERIMENTAL

All reagents were USP, NF, or ACS grade. Spectroquality 2-propanol was purchased. Samples of commercial progesterone injection NF were obtained locally and from manufacturers. Fixed oils were obtained locally.

Chromatographic System—The instrument[1] used a 1-m by

[1] DuPont model 830 high-pressure liquid chromatograph, fitted with circulating warm-air oven and low-pressure mercury UV photometer.