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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS–LIQUID CHROMATOGRAPHY OF SOME NORGESTREL INTERMEDIATES

PHYSICAL PROPERTIES OF THE ISOLATED *SYN*- AND *ANTI*-ISOMERS OF OXIMES

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SUMMARY

High-performance liquid chromatographic (HPLC) and gas–liquid chromatographic (GLC) separations of some steroidal α,β-unsaturated ketoximes, intermediates in the synthesis of the oral contraceptive norgestrel, were investigated. When GLC (trimethylsilyl derivatives) or reversed-phase HPLC was used, the *syn*- and *anti*-isomers were not resolved. However, with the *syn*/*anti* ratio changing markedly in the samples, these methods (especially reversed-phase HPLC) proved to be less accurate because the physical properties relevant to detection were different for the isomers examined. Improved accuracy can be achieved by using normal-phase HPLC and taking the corresponding isolated *syn*- and *anti*-isomers as reference standards.

INTRODUCTION

The last steps in the synthesis of the steroidal contraceptive norgestrel involve the following intermediates[1] (see Fig. 1): 13β-ethyl-3-methoxygona-2-5(10)-dien-17β-ol (i), 13β-ethyl-3-oximinogon-4-en-17β-ol (ii), 13β-ethyl-3-oximinogon-4-en-17-one (iii) and 13β-ethyl-17α-ethynyl-3-oximinogon-4-en-17β-ol (iv). The oxime intermediates (ii, iii and iv) appear as a mixture of *syn*- and *anti*-isomers. As determinations of melting points and optical rotation are of minor value in such instances, separation methods were needed in order to give analytical support to the elaboration of the synthesis.

In the past, steroids have been separated and quantitated by paper, thin-layer, gas and column chromatography, but few papers, using thin-layer chromatography (TLC)[2,3] and reversed-phase high-performance liquid chromatography (HPLC)[4], have dealt with the separation of steroidal oximes.

As pointed out by Hara *et al.*[3], the *syn*- and *anti*-isomers of testosterone oxime showed a 44% difference in their molar absorptivities at 242 nm, and other steroidal α,β-unsaturated ketoximes of the 4-en-3-one or 1-en-3-one series also differed significantly in their physical properties.

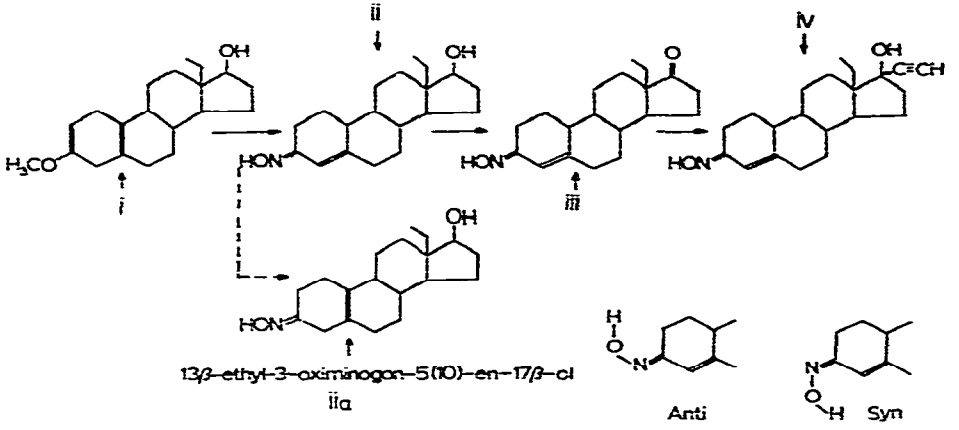


Fig. 1. Part of the synthesis of norgestrel.

Huettemann and Shroff[4] reported the determination of 17α-ethynyl-17β-acetoxy-19-norandrost-4-en-3-one oxime by reversed-phase HPLC using ultraviolet (UV) detection at 254 nm. With the isomers unresolved and having Hara *et al.*'s results in mind, issue can be taken with the accuracy of the reversed-phase method, and with that of reversed-phase HPLC methods in general, for the quantitative determination of such oximes by UV detection, when the *syn*/*anti* ratio in the samples relative to this ratio in the reference standard changes.

This paper deals with the separation and determination of some gon-4-en-3-one oximes (ii, iii and iv) by GLC and HPLC and the determination of some physical parameters of the isolated *syn*- and *anti*-isomers.

EXPERIMENTAL

Gas–liquid chromatography

GLC separations were carried out with an HP 5830 A instrument equipped with a flame-ionization detector (FID). The columns were 6 ft. × 4 mm I.D. glass coils with 3% OV-1 or 2% OV-17 on 80–100-mesh Gas-Chrom Q (phases and support material from Applied Science Labs., State College, Pa., U.S.A.). The temperature of the injection port, the column and the detector was 240°. The carrier gas (nitrogen) flow-rate was 46 ml/min, the chart speed was 0.6 cm/min and 1-μl solutions were injected. Cholestane (10 μg) was used as the internal standard.

Preparation of derivatives

Trimethylsilyl (TMS) derivatives were prepared with 0.1–0.2 mg of steroid and with 120 μl of reagent mixture [bistrimethylsilyltrifluoracetamide (BSTFA)–trimethylchlorosilane (TMCS)–pyridine (PY) (4:1:1) (reagents from Pierce, Rockford, Ill., U.S.A.)] at 60° for 1 h.

Methyloxime (MO) derivatives were prepared with 0.1–0.2 mg of steroid and with 100 μl of O-methyloxyammonium chloride (Pierce) in pyridine (16 mg/ml) at 60° for 3 h.

When preparing the MO-TMS derivatives, we first followed the procedure

described for the MO derivatives. After the evaporation of the excess of pyridine under nitrogen, 100 μl of BSTFA–PY (4:1) solution was added to the crude adduct and the mixture was kept at 60° for 1 h.

Mass spectrometry

Compounds ii, iia, iii and iv were analysed by GLC–MS as their TMS, MO and MO-TMS derivatives. An HP 5990 A gas chromatograph–mass spectrometer was used. The column was a 2 ft. × 2 mm I.D. glass coil packed with 2% OV-101 on 100–120-mesh Gas-Chrom Q. The electron energy was 70 eV and the multiplier voltage was 2600 eV.

High-performance liquid chromatography

A Varian 8500 HPLC pump (with stop-flow septumless injection) was used, equipped with a variable-wavelength UV–VIS detector (Variscan, at 240 nm) and a Model A-25 strip-chart recorder for the analytical work, and with an ISCO Model UA-5 (Type 6) absorbance monitor (254 nm, with peak separator and built-in recorder), as well as a Model 328 fraction collector, for the HPLC isolation of the *syn*- and *anti*-isomers. The HPLC columns (packings and dimensions) used are indicated in Table IV. Micro-Pak columns were supplied by Varian (Palo Alto, Calif., U.S.A.) and the remainder of the pre-packed columns were purchased from Chrompack (Middelburg, The Netherlands). Chloroform, isooctane, isopropanol and dioxane (Chrom AR grade) were products of Mallinckrodt, (St. Louis, Mo., U.S.A.), and acetonitrile, *n*-hexane (Uvasol), dichloromethane, ethyl acetate (Li-Chrosolve) and methanol (Selectipur) were obtained from Merck (Darmstadt, G.F.R.). Other chemicals used were of the highest grade available.

HPLC analytical determinations were carried out using system V or VII (see Table IV), with an eluent flow-rate of 100 ml/h and pure norgestrel as the internal standard (mostly 10 μg/ml). The injection volume was 20 μl. Samples and the internal standard were dissolved either in the eluent or in ethyl acetate–isooctane mixtures.

For the isolation of the *syn*- or *anti*-isomers in repetitive runs, we chose low-boiling solvents (system V or III, the latter having been used with pure ii and pure iv mixtures), injecting 40 μl of a solution containing 1 mg of isomer mixture in ethyl acetate–methanol (9:1) each time. Collected fractions containing the isomer of interest were evaporated to dryness at reduced pressure and ambient temperature. The crystals obtained were weighed and stored protected from heat and light and used as reference standards when needed. Stock solutions of a reference standard were prepared by dissolving small amounts of the crystalline material in ethyl acetate to give solutions of 1 mg/ml and adding an equal volume of isooctane. If stored in a refrigerator, the isomers were stable in stock solutions for at least 1 week. When preparing a calibration graph, additional dilutions of the stock solutions were prepared with isooctane.

UV, infrared and nuclear magnetic resonance spectra and melting points

UV spectra were taken in ethanol on a Cary Model 118 C UV–VIS spectrophotometer, and infrared (IR) spectra were recorded with a Perkin-Elmer 577 instrument (potassium bromide pellets). For the nuclear magnetic resonance (NMR) analysis of the iii oximes a JEOL HL-60 spectrometer was used with $CDCl_3$ as the

solvent, at room temperature, and with tetramethylsilane as the internal standard. Melting points were determined with a thermomicroscope (Boetius system).

RESULTS AND DISCUSSION

In order to determine optimal chromatographic conditions for the separation of compounds ii, iii and iv, GLC and HPLC studies were carried out.

Gas–liquid chromatography

As compounds ii, iii and iv decompose easily at higher temperatures, sufficiently volatile and stable derivatives had to be prepared.

We prepared 3,17-di-TMS and 3-MO-17-TMS derivatives of compounds ii and iv, and 3-TMS and 3,17-di-MO derivatives of compound iii.

As can be seen in Fig. 2, the TMS derivatives of the *syn/anti* isomers of ii, iii and iv were not resolved under the chosen conditions and were eluted as single peaks. On the other hand, MO-TMS derivatives of compounds ii and iv (and di-MO derivatives of iii) eluted in double peaks, indicating a tendency for separation and resulting in poorer quantitation possibilities (see Fig. 3). Thus, for reasons of easier quantitation, we prepared the TMS derivatives and first examined the stability of these derivatives under various derivatization conditions.

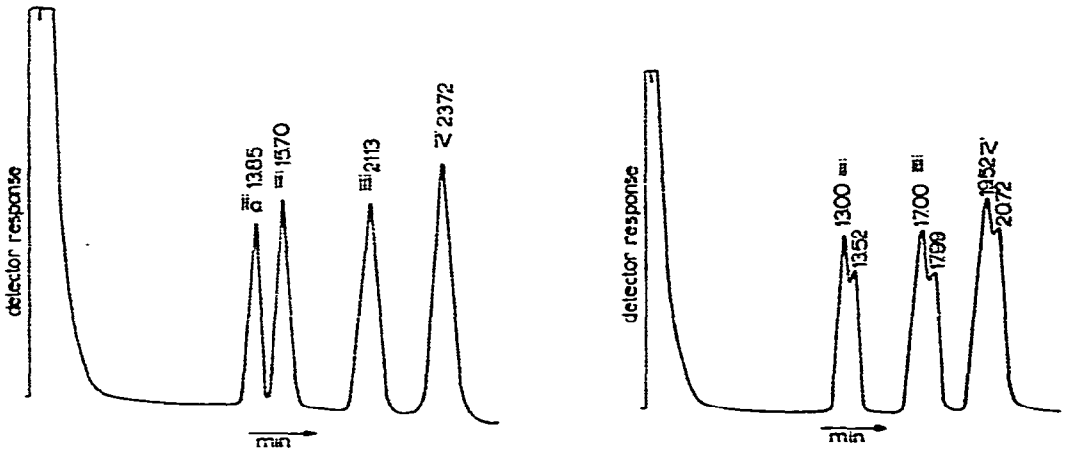


Fig. 2. Typical gas chromatogram for TMS derivatives of compounds ii, iia, iii and iv on a 2% OV-17 6 ft. × 4 mm I.D. glass coil column. Column temperature: 230°.

Fig. 3. GLC of MO-TMS derivatives of compounds ii, iv and the di-MO derivative of compound iii. Conditions as in Fig. 2.

With steroids containing ketone groups, the formation of enol–TMS ether derivatives is possible during silylation, as reported on by Chambaz *et al.*[5]. However, enol ether formation is less likely with 17-one steroids[5].

Compound iii was subjected to prolonged silylation, for 24 h in BSTFA–TMCS–PY (4:1:1) at 60°, but no enol ether was formed even when pyridine was replaced with acetonitrile, tetrahydrofuran or dimethylformamide. As a consequence, there was no need to prepare MO-TMS derivatives.

TMS derivatives of compounds ii, iii and iv were stable under the operating conditions reported, as indicated by the linear calibration graphs obtained.

When compound i was converted into compound ii, an additional peak (iia, Fig. 2) appeared besides ii in the gas–liquid chromatogram (TMS). Having run the mass spectra of components ii and iia, we found identical peaks in the spectra of ii and iia, differing only in relative intensities (see Table I). The fact that iia is a Δ^5 isomer of ii was comfirmed by comparing the GLC data and GLC–MS results for synthetically prepared 13β-ethyl-3-oximinogon-5(10)-en-17β-ol with those of component iia.

Table I gives the GLC–MS data for the TMS, MO-TMS and di-MO derivatives of the compounds examined. These data confirmed the formation of the derivatives studied.

TABLE I

RELATIVE ABUNDANCES OF PRINCIPAL FRAGMENTS OF TMS, MO-TMS AND DI-MO DERIVATIVES OF COMPOUNDS ii, iia, iii AND iv AT 70 eV

Notations: 1 = $[M - {}^{\bullet}CH_3]^+$; 2 = ring A + C_6; 3 = $[(C_{15}–C_{17}) - {}^{\bullet}H]^+$; 4 = $[M - 90]^{+\bullet}$; 5 = $[M - 2 \times 90]^{+\bullet}$; 6 = $[M - C_{16}–C_{17}]^{+\bullet}$; 7 = $[M - {}^{\bullet}CH_3CH_2]^{+\bullet}$; 8 = $[M - {}^{\bullet}OCH_3]^+$; 9 = $[M - 90 - {}^{\bullet}CH_3]^+$.

Compound	*Molecular ion and relative intensity*	*Base peak*	*Masses and relative intensities of some fragments*
ii (3,17-di-TMS)	447 (59)	73	432^1 (15), 197^2 (12), 129^3 (30)
ii (3-MO-17-TMS)	389 (82)	73	358^8 (17), 299^4 (14), 268^9 (30)
iia (3,17-di-TMS)	447 (4)	73	432^1 (10), 197^2 (10), 357^4 (52), 267^5 (15), 129^3 (39)
iii (3-TMS)	373 (99)	358^1	197^2 (10), 73 (55)
iii (3,17-di-MO)	344 (70)	313^8	73 (14)
iv (3,17-di-TMS)	471 (20)	331^6	153^3 (24), 456^1 (11), 442^7 (25)
iv (3-MO-17-TMS)	413 (33)	73	398^1 (9), 382^8 (10), 323^4 (7), 273^5 (37)

In order to make the qualitative identification more reliable, we determined the net retention times and retention indices of derivatized ii, iia, iii and iv on OV-1 and OV-17 stationary phases, as shown in Table II.

Although the GLC method was applicable to the determination of compounds ii, iii, iv, i and iia (the last two being difficult to quantitate by HPLC), in our hands there were slight changes in the slopes of the calibration graphs whenever reference standards with significantly different *syn/anti* ratios (the ratio having been determined by HPLC) from that in earlier reference standards were used. Consequently, when the *syn/anti* ratio in a sample and in the respective reference standard differed greatly, calculations led to more or less inaccurate results. In such instances we used the relative molar response (RMR) method for the quantitation of the successive intermediates instead[6].

TABLE II

NET RETENTION TIMES AND RETENTION INDICES OF TMS DERIVATIVES OF COMPOUNDS ii, iia, iii AND iv ON OV-1 AND OV-17 STATIONARY PHASES

Compound	*OV-1*		*OV-17*	
	*Net retention time, t'_R (min)**	*Retention index*	*Net retention time, t'_R (min)***	*Retention index*
ii (3,17-Di-TMS)	18.50	2953	15.20	3019
iia (3,17-Di-TMS)	16.10	2900	13.37	2976
iii (3-TMS)	15.10	2875	20.63	3122
iv (3,17-Di-TMS)	24.80	3059	23.23	3161

* t'_R data of hydrocarbons (min): $C_{28} = 12.32$; $C_{29} = 16.10$; $C_{30} = 21.16$; $C_{31} = 27.66$.
** t'_R data of hydrocarbons (min): $C_{29} = 10.64$; $C_{30} = 14.38$; $C_{31} = 19.33$; $C_{32} = 26.00$.

On the basis of GLC (TMS) studies with HPLC-isolated *syn*- and *anti*-isomers we found that these isomers exhibited identical retention properties but appear to give different FID responses. Our efforts to elucidate the cause of this anomaly were unsuccesful, as even single isomers were converted into a *syn/anti* equilibrium mixture during the derivatization process, as was evidenced by the double peaks obtained after MO derivatization of single isomers of ii, iii or iv.

High-performance liquid chromatography

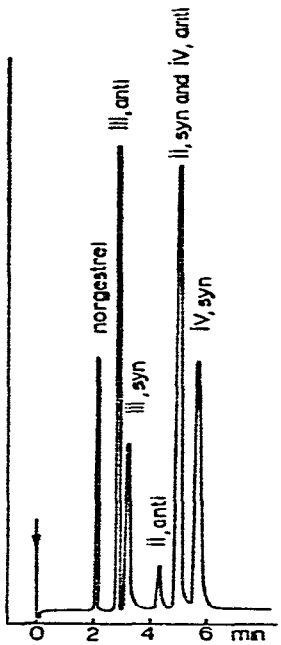
The principal UV absorber in steroidal α,β-unsaturated ketoximes is the –N=C–C=C– π-electron system. As the lone pair of electrons on the oxygen atom in the oximino group of a *syn*-isomer may interact, to some extent, with the chromophoric system (through H-4 in 4-en-3-one oximes), whereas those in an *anti*-isomer cannot, there is usually some difference in the region and/or intensity of the UV absorption of such isomers, as pointed out by Hara *et al.*[3]. Thus, the accuracy of an HPLC method with UV detection has to be treated with caution if the *syn/anti*-isomers of such oximes are unresolved (*e.g.*, in normal reversed-phase HPLC[4] and in system IX, see Tables III and IV).

Huettemann and Shroff[4] found a variation in the slope of the Beer's law plot "from time to time due to the quality of the solvent and/or temperature". The cause of the deviation may have been a change in the *syn/anti* ratio in the oximes used as reference standards, as this ratio can easily be influenced by an increase in the temperature and/or a change in the quality of the solvent.

Our aim was to isolate the pure isomers and to use each of them as reference standard for the corresponding component in a mixture.

Some of the HPLC systems studied are shown in Tables III and IV. The best separations were achieved using systems V (the "dryest" of the silica systems), VII and VIII (systems with a CN-bonded silica packing). In each pair, *anti*-isomers were eluted first, in good agreement with TLC separation studies on other steroidal oxime isomers of this type[3]. Systems II and III exhibited a greater selectivity towards *syn*-isomers, all three being eluted after the *anti* group. System VII, the quickest of the suitable systems, was used whenever compound ii in compound iii or compound iii in compound iv was only to be examined (ii, *syn* and iv, *anti* are not separated in this system; see Fig. 4). System V was used for preparative purposes and also in

analytical examinations when a sample containing all six isomers had to be analysed (see Fig. 5).



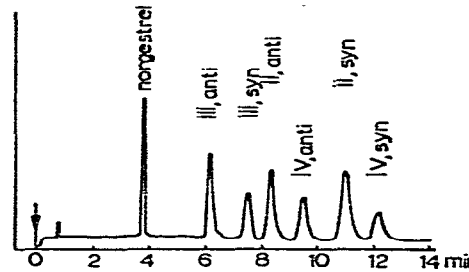


Fig. 4. Separation of a test mixture of some norgestrel intermediates in system VII (see Table IV).
Fig. 5. Separation of the *syn*- and *anti*-isomers of compounds ii, iii and iv in system V (see Table IV).

Table V gives some physical properties of the pure isolated *syn*- and *anti*-isomers of ii, iii and iv. All three *anti*-isomers (the assignment of configurations having been made by NMR spectroscopy, from the chemical shifts of the olefinic proton) showed stronger UV absorption maxima at shorter wavelengths than the corresponding *syn*-isomers. At 240 nm there was a 41% difference between the UV absorption intensities of the *syn*- and *anti*-isomers of compound iv, a 21% difference between those of compound ii and a 19% difference between those of compound iii.

These data indicate the extent to which analytical results may be inaccurate when extreme changes in the *syn/anti* ratio occur and reversed-phase HPLC with UV detection is used.

UV absorption bands of all three *syn*-isomers were broader than those of the corresponding *anti*-isomers, as indicated by the molar absorptivities at 280 nm. If combined with chromatographic data, both the UV absorption value at this wavelength and the ratio of the IR band intensities at 860 and 890 cm^{-1} (the ratio being less than 0.75 for *syn*-isomers and greater than 1,20 for *anti*-isomers) can be used as a means of distinguishing between *syn*- and *anti*-gon-4-en-3-one oxime isomers.

When isolating the isomers we used system III or V, depending on the composition of the starting mixture, and collected only central cuts of peaks in order to avoid cross-contamination.

TABLE III

HPLC SYSTEM SELECTIVITY STUDIES WITH SOME GON-4-EN-3-ONE OXIME ISOMERS

For compositions of systems, see Table IV.

Compound		*System (eluents, v/v)*																	
		I		*II*		*III*		*IV*		*V*		*VI*		*VII*		*VIII*		*IX*	
		k'	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*	*k'*	*α*
Norgestrel										4.70				3.16		3.78			
iii (17-one)	*anti*	1.90	1.14					6.05	1.20	7.25	1.23	2.96	1.61	4.62	1.12	5.93	1.17	5.46	
	syn	2.17						7.26		8.95		4.76		5.18		6.92		5.46	1.22
ii (17-ol)	*anti*	2.26	1.04					8.00	1.10	10.12	1.13	4.85	1.02	7.28	1.40	7.68	1.11	4.45	
	syn	4.40	1.94					10.20	1.28	13.60		7.94	1.64	8.43	1.16	9.03	1.18	4.45	1.21
iv (17-ethynyl)	*anti*	4.63	1.05					10.60	1.04	11.52	1.18	8.17	1.03	8.71	1.03	11.00	1.22	3.68	
	syn	5.20	1.12					12.40	1.17	15.80		9.32	1.13	10.00	1.15	13.08	1.19	3.68	
iii	*anti*			2.65	1.16	6.25	1.05												
ii	*anti*			3.08	1.08	6.57	1.03			10.12	1.14								
iv	*anti*			3.32	1.07	6.68	1.10			11.52									
iii	*syn*			3.56	1.12	7.35	1.12												
ii	*syn*			4.00	1.07	8.23	1.05			13.60	1.12								
iv	*syn*			4.30		8.65				15.80									

TABLE IV

HPLC SYSTEMS I–IX USED AND INDICATED IN TABLE III

W = water saturated.

I	*II*	*III*	*IV*	*V*
Partisil 5, 250 × 4.6 mm I.D.		Spherisorb S 10 W, 250 × 3 mm I.D.		
n-Hexane 30 Chloroform 50 Acetonitrile 19 Isopropanol 1	*n*-Hexane 50 (W) *n*-Hexane 25 Dioxane 20 Acetonitrile 5	*n*-Hexane 40 *n*-Hexane 40 Dichloromethane 14 } (W) Acetonitrile 6 Ammonia, 25% (w/v) 0.02	*n*-Hexane 45 *n*-Hexane 40 (W) Dichloromethane 9 Acetonitrile 6 Methanol 0.2 Ammonia, 25% (w/v) 0.02	*n*-Hexane 85 Chloroform 9 Acetonitrile 6 Methanol 0.08 Ammonia, 25% (w/v) 0.02

VI	*VII*	*VIII*	*IX*
Micro-Pak CN-10, 250 × 2 mm I.D.	Sil 60D 5CN, 150 × 3 mm I.D.		Micro-Pak CH-10, 300 × 4 mm I.D.
Isooctane 40 *n*-Hexane 45 Chloroform 10 } (W) Acetonitrile 5	Isooctane 40 *n*-Hexane 45 Chloroform 10 } (W) Acetonitrile 5	Isooctane 41 *n*-Hexane 45 Chloroform 6 Acetonitrile 7 Isopropanol 1	Acetonitrile 60 0.01 *M* $(NH_4)_2CO_3$ in water 40

TABLE V

PHYSICAL PROPERTIES OF PURE ISOLATED *SYN*- AND *ANTI*-ISOMERS OF SOME GON-4-EN-3-ONE OXIMES

Compound		*Melting point (°C)*	*UV*			*IR: band intensity ratio, I (860 cm⁻¹) / I (890 cm⁻¹)*	*NMR: δH − 4 ppm (H–C=C)*
			$\lambda_{max.}^{EtOH}$ *nm*	*log ε*	*ε at 280 nm (l/mole·cm)*		
ii (17-ol)	*anti*	191–193	239	4.26	0.0	1.32	
	syn	180–182	242	4.16	2920	0.28	
iii (17-one)	*anti*	202–203	238	4.42	0.0	1.22	5.85
	syn	176–178	240	4.33	4650	0.52	6.53
iv (17-ethynyl)	*anti*	128–130	239	4.22	0.0	1.93	
	syn	134–136	242	3.99	2060	0.74	

The kinetics of interconversion was studied in crystalline form and in milligram per millilitre solutions in methanol, acetone, chloroform, ethyl acetate and ethyl acetate–isooctane mixtures at various temperatures. We found that elevated temperatures should always be avoided. In methanol, isomerization of ii, iii or iv oximes occurs quickly even at room temperature. An isomer solution on standing in acetone at room temperature gave a small amount of the corresponding isomer within 5 h. In crystalline form and in ethyl acetate or in ethyl acetate–isooctane mixtures each isomer was stable. Stock solutions in the latter mixture can be used for at least 7–10 days if stored protected from heat and light.

In system V the detection limits of compounds iii (*anti*), iii (*syn*), ii (*anti*), ii (*syn*), iv (*anti*) and iv (*syn*) were 5, 7, 10, 18, 15 and 33 ng, respectively. The precision ranged from $\pm$ 1.5% to $\pm$ 3.5% and was highest for iii (*anti*) and lowest for iv (*syn*), decreasing in the order of elution.

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