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STUDIES ON STEROIDS

CIX. O-ω-HALOALKYLOXIMES, NEW DERIVATIVES FOR GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF OXOSTEROIDS

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SUMMARY

Several kinds of ω-haloalkoxyamines have been prepared as new derivative-forming agents applicable for the gas chromatography–mass spectrometry of carbonyl compounds. Of these reagents, 2-chloroethoxyamine hydrochloride [O-(2-chloroethyl)hydroxylamine] was the most suitable for use with oxosteroids because of its higher reactivity and the appropriate retention value and satisfactory chromatographic properties of the resulting oximes. The contribution to the retention value due to formation of the 2-chloroethyloxime was determined for 5α-androstanones having the oxo-group at various positions in the steroid nucleus. As expected, 17-oxosteroid O-2-haloethyloximes exhibit readily distinguishable isotope peaks as a cluster in the mass spectrum.

INTRODUCTION

In recent years, considerable attention has been directed to the development of derivatizing reagents for use in gas chromatography (GC). With regard to carbonyl compounds, a variety of derivatizing agents such as alkoxyamines and alkyl- or arylhydrazines is now widely used[1–15]. In a previous paper in this series, O-pentafluorobenzylhydroxylamine was proposed as a sensitive derivatizing reagent for use in GC with electron-capture detection[16]. Characterization of trace amounts of steroid hormones and drug metabolites in biological fluids by means of GC–mass spectrometry (GC–MS) requires that the ion peaks derived from these compounds should be relatively easily distinguishable from those due to contamination. Recently, Chapman and Bailey reported the suitability of halomethyldimethylsilyl ethers as derivatives for determining hydroxylated steroids at low levels by a multiple peak-monitoring technique[17,18]. The presence of chlorine or bromine in a molecule provides a charac-

teristic pattern exhibiting the isotope peaks as a cluster in the mass spectrum. However, a carbonyl-group reagent yielding a chloro- or bromo-substituted derivative has not yet been reported for this purpose. This paper deals with the preparation of ω-haloalkoxyamines as new derivatizing reagents and their application to the GC–MS of 17-oxosteroids.

EXPERIMENTAL

Preparation of derivative-forming reagents

N-(ω-Chloroalkoxy)phthalimides (IIa–c). To a stirred solution of N-hydroxyphthalimide (I)[19] (0.05 mole) and triethylamine (30 ml) in dimethylformamide (30 ml) was added an α,ω-dichloroalkane (0.2 mole), and the mixture was heated at 80° for 5 h. The precipitate was then removed by filtration, the filtrate was poured into ice–water, and the resulting precipitate was filtered off and recrystallized from aqueous ethanol to give II in 55–70% yield.

ω-Chloroalkoxyamine hydrochlorides (IIIa–c). A solution of II (0.04 mole) in 60 ml 6 *N* hydrochloric acid–glacial acetic acid (1:1) was heated at 100° for 5 h. The resulting solution was evaporated *in vacuo*, and the residue was diluted with 15 ml water and allowed to stand in a refrigerator. After removal of the precipitate by filtration, the filtrate was evaporated to dryness and the residue was kept in a desiccator overnight; the pale brown cake thus obtained was recrystallized from ethanol to give III in 20–35% yield.

2-Bromoethoxyamine hydrobromide. This compound was prepared by condensation of N-hydroxyphthalimide with 1,2-dibromoethane, followed by hydrolysis with hydrobromic acid in acetic acid as described by Bauer and Suresh[20]. Recrystallization of the crude product from ethanol gave 2-bromoethoxyamine hydrobromide as colourless leaflets (m.p. 183–184°; literature value 185–187°).

Steroids. Dehydroepiandrosterone was donated by Teikoku Hormone Mfg. Co. (Tokyo, Japan); 5α-androstanones were prepared in these laboratories by accepted procedures[21].

Methods

Gas chromatography. The apparatus used for this work was a Shimadzu model GC-4BMPFE gas chromatograph equipped with hydrogen-flame ionization and ^{63}Ni electron-capture detectors and a U-shaped glass column (2 m × 3 mm I.D.). The column was packed with 2% of SE-30 on Gas-Chrom Q (80–100 mesh). The detector and flash heater were kept at 240°, and the column temperature was 220°. Nitrogen was used as carrier gas at a flow-rate of 55 ml/min. The relative retention time of each sample was measured with cholestane as reference compound. According to the definition proposed by VandenHeuvel and Horning[22], a plot of the logarithm of the relative retention time against "steroid number" (SN) was made, the values of androstane and cholestane being taken as 19 and 27, respectively.

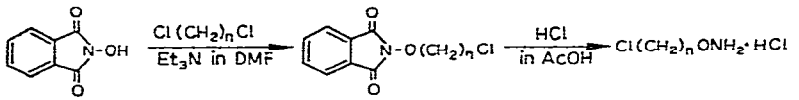
Derivatization procedure. To a solution of the oxosteroid (*ca.* 0.1 mg) in pyridine (1 drop) was added ω-chloroalkoxyamine hydrochloride (or 2-bromoethoxyamine hydrobromide) (2 mg), and the mixture was heated at 100° for 1 h. The reaction mixture was diluted with hexane (1 ml), then washed successively with water (1 ml), 0.1 *N* hydrochloric acid (1 ml) and water (1 ml). After evaporation of the organic

layer with the aid of a stream of nitrogen, the residue was treated with hexamethyldisilazane (0.1 ml) and chlorotrimethylsilane (0.1 ml) in pyridine (5 drops) according to the procedure of Sweeley *et al.*[23]. After the usual work-up, the residue was dissolved in hexane (2 drops) and injected into the gas chromatograph.

Gas chromatography–mass spectrometry. A Shimadzu model LKB-9000 gas chromatograph–mass spectrometer equipped with a data-processing system was used. The coiled glass column (1 m × 2.5 mm) was packed with 1.5% of OV-101 on Gas-Chrom Q (80–100 mesh), and the flow-rate of carrier gas (helium) was 30 ml/min. The temperature of column was 230°, and the injection port and ion source were kept at 270°. The accelerating voltage, ionization voltage and ionization current were 3.5 kV, 70 eV and 60 μA, respectively.

RESULTS AND DISCUSSION

An initial effort was directed to preparation of the O-2-chloroethyl-, -4-chlorobutyl-, -5-chloropentyl- and -2-bromoethyl-derivatives of hydroxylamine, whose halogen is responsible for the occurrence of the isotope peaks in the mass spectra. Condensation of N-hydroxyphthalimide (I) with an excess amount of α,ω-dihaloalkane proceeded easily in the presence of triethylamine, resulting in the formation of N-(ω-haloalkoxy)phthalimides (IIa–c); subsequent hydrolysis with hydrochloric acid provided the desired O-ω-haloalkylhydroxylamine hydrochlorides (IIIa–c) in reasonable yield. The properties and analytical data for these compounds are listed in Tables I and II.



I II III

a: n=2; b: n=4; c: n=5

Derivatization of the 17-oxosteroid with ω-haloalkoxyamines was then attempted. It was found that 2-chloroethoxyamine condensed with the ketone in pyridine under the mild conditions to provide the oxime in quantitative yield, but other homologous reagents were somewhat less reactive. The increment in retention value due to transformation into the oxime by means of these reagents was determined, the 3-trimethylsilyl ether of dehydroepiandrosterone being used as reference; the relative retention times and SN values are collected in Table III, which shows that the retention value of the parent ketone was significantly increased by the bulky substituent introduced at C-17. In consideration of its higher reactivity and the appropriate retention time of the derivative, 2-chloroethoxyamine was chosen as being the most suitable derivatizing reagent.

The SN contributions due to conversion into the O-2-chloroethyloxime were then determined for 5α-androstanones having the oxo-group at various positions. The resulting oximes gave peaks of the theoretical shape, indicating satisfactory GC properties. As can be seen from Table IV, the increment in SN due to derivatization varies from 3.4 to 4.5 with the location of the oxo-group. Almost all the compounds showed a single peak, except for the 5α-androstan-2-one derivative, which exhibited

TABLE I

PROPERTIES AND ANALYTICAL DATA OF N-(ω-CHLOROALKOXY)PHTHALIMIDES

Compound	*n*	*M.p. (°C)*	*Appearance*	*Formula*	*Analysis (%)*					
					Calculated			*Found*		
					C	*H*	*N*	*C*	*H*	*N*
IIa	2	96–98	Colourless needles	$C_{10}H_8ClNO_3$	53.23	3.57	6.21	53.40	3.84	6.06
IIb	4	55–56	Colourless needles	$C_{12}H_{12}ClNO_3$	56.81	4.77	5.52	56.68	4.82	5.49
IIc	5	73.5–74	Colourless needles	$C_{13}H_{14}ClNO_3$	58.32	5.27	5.23	58.35	5.38	5.17

TABLE II

PROPERTIES AND ANALYTICAL DATA OF ω-CHLOROALKOXYAMINE HYDROCHLORIDES

Compound	*n*	*M.p. (°C)*	*Appearance*	*Formula*	*Analysis (%)*					
					Calculated			*Found*		
					C	*H*	*N*	*C*	*H*	*N*
IIIa	2	122–125	Colourless leaflets	$C_2H_7Cl_2NO$	18.02	5.35	10.61	18.32	5.38	10.68
IIIb	4	130–132	Colourless leaflets	$C_4H_{11}Cl_2NO$	30.02	6.93	8.75	30.51	6.89	8.71
IIIc	5	90– 91	Colourless leaflets	$C_5H_{13}Cl_2NO$	34.50	7.53	8.05	34.53	7.53	8.39

TABLE III

RELATIVE RETENTION TIMES (RRT) AND STEROID NUMBERS (SN) OF DEHYDROEPIANDROSTERONE 17-(O-ω-HALOALKYL)OXIME 3-TRIMETHYLSILYL ETHERS

For experimental details, see *Methods*.

Compound	*RRT*	*SN*	*ΔSN**
Dehydroepiandrosterone 3-trimethylsilyl ether	0.47	24.3	
17-(2-Chloroethyl)oxime	1.56	28.5	4.2
17-(4-Chlorobutyl)oxime	2.96	30.7	6.4
17-(5-Chloropentyl)oxime	4.06	31.8	7.5
17-(2-Bromoethyl)oxime	2.00	29.4	5.1
Cholestane	1.00	27.0 (15.1 min)	
Androstane	0.10	19.0 (1.6 min)	

* Expressed as an increment to SN observed when dehydroepiandrosterone 3-trimethylsilyl ether was converted into the oxime.

two peaks (probably due to the formation of *syn*- and *anti*-isomers). The 11-oxosteroid, which is to some extent sterically hindered, did not react with the reagent under the conditions employed.

The increment in SN produced when the parent ketone is converted into the oxime derivative appears to be characteristic of the position on the steroid nucleus; the steric effect involving the functional group probably contributes to the SN increment to a lesser extent. Derivatization seems to be effective in magnifying differences due to the position of the oxo-group on the steroid skeleton, as exemplified by the

TABLE IV

STEROID NUMBERS (SN) OF 5α-ANDROSTANONE O-2-CHLOROETHYLOXIME DERIVATIVES

For experimental details, see *Methods*.

Position of functional group	*SN*	*SN contribution*		*ΔSN***
		Oxime	*Ketone**	
1	24.4	5.4	1.8	3.6
2	25.0	6.0	2.2	3.8
	25.3	6.3		4.1
3	25.8	6.8	2.5	4.3
4	25.3	6.3	2.1	4.2
6	24.8	5.8	2.1	3.7
7	24.3	5.3	1.9	3.4
15	24.8	5.8	1.9	3.9
16	25.7	6.7	2.2	4.5
17	25.3	6.3	2.2	4.1

* See ref. 21.

** Expressed as an increment to SN observed when the parent ketone was converted into the oxime.

fact that the 4- and 6-oxosteroids are distinctly separated when transformed into the oximes. On conversion into oxime derivatives, positional 16-oxo- and 17-oxo-isomers may possibly be separated on the usual phase.

Examinations were then made on the sensitivity of the O-ω-haloalkyloximes for GC with electron-capture detection. Dehydroepiandrosterone O-2-chloroethyloxime was found to be several times as sensitive as the parent ketone, but less than expected. Also, the homologous O-ω-haloalkyloximes showed sensitivities almost identical with that of the O-2-chloroethyloxime.

The mass spectra of dehydroepiandrosterone 17-(O-2-haloethyl)oxime 3-trimethylsilyl ethers are illustrated in Fig. 1. The presence of chlorine or bromine in the molecule provides a characteristic isotopic pattern. Hence, the ratio of intensities at *m/e* 437 (^{35}Cl) and 439 (^{37}Cl), or at *m/e* 481 (^{79}Br) and 483 (^{81}Br), can serve as a check on the absence of contributions from other materials at these masses. In the mass spectrum of dehydroepiandrosterone O-2-chloroethyloxime trimethylsilyl ether, an intense peak at *m/e* 308 (M−129) is due to the elimination of the trimethylsilyl group and three carbon atoms of ring A from the molecular ion; this is a characteristic fragmentation sequence for the trimethylsilyl ether of Δ^5-3-hydroxysteroids[24]. The fragment ions at *m/e* 347 (M−90) and 422 (M−15) result from loss of the trimethylsilanol and methyl group, respectively[25]. A similar fragmentation pattern is observed in the mass spectrum of the O-2-bromoethyloxime.

The potential utility of ω-haloalkoxyamines as derivatizing reagents in GC–MS may be helpful for the analysis of ketones, in particular, oxosteroids and drug metabolites, in biological fluids.

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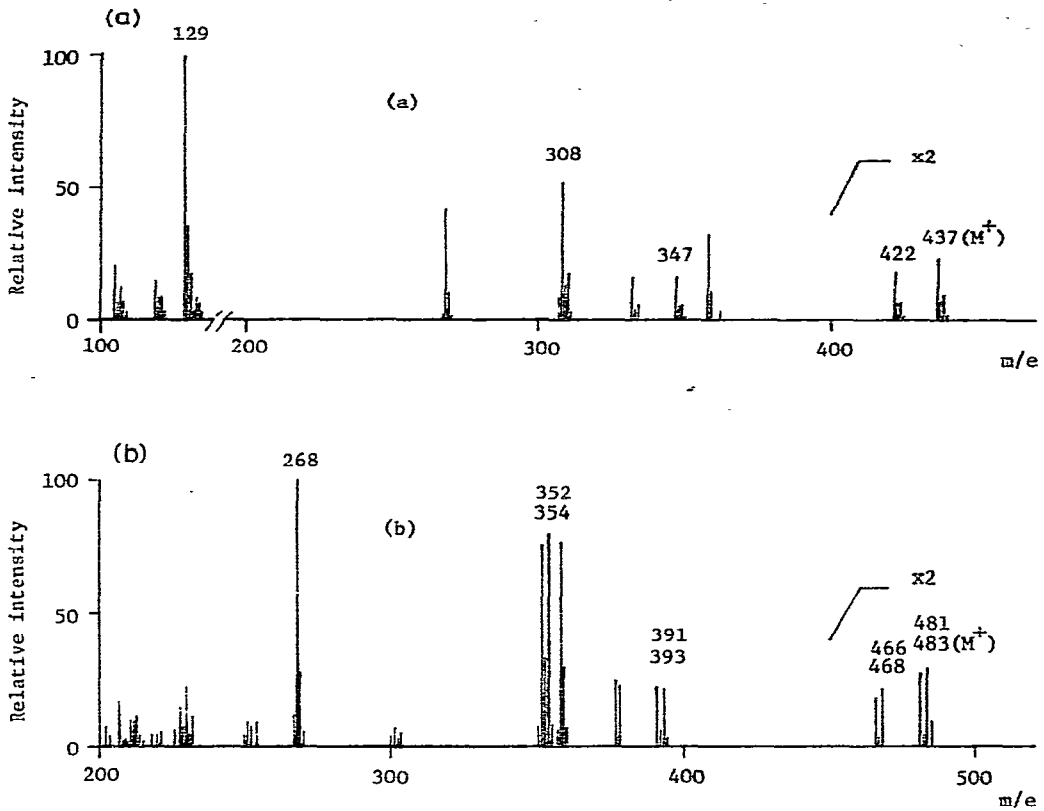

Fig. 1. Mass spectra of 17-(O-2-chloroethyl)oxime (a) and 17-(O-2-bromoethyl)oxime (b) of dehydroepiandrosterone 3-trimethylsilyl ether.

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