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A HIGHLY LIPOPHILIC FORM OF ALDOSTERONE. ISOLATION AND CHARACTERIZATION OF AN ALDOSTERONE DIMER

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Summary—A highly lipophilic form of aldosterone obtained both from incubations of 18-hydroxycorticosterone with quartered rat adrenals and by treatment of aldosterone with acid, was identified as an aldosterone dimer based on its ^{1}H, ^{13}C NMR and mass spectra.

INTRODUCTION

The incubation of 18-hydroxycorticosterone (18-OH-B, M form‡) with quartered rat adrenals at different pHs (7.4, 4.8, 3.3) [1] yields aldosterone plus several other compounds that may be partially separated by paper chromatography in the Bush B5 solvent system [2]. In the fraction corresponding to the lower polarity components (R_f 0.93) we were able to identify three compounds, two of which could be hydrolysed back to 18-OH-B (M form). We here report the identification and characterization of the third compound as a highly lipophilic form of aldosterone (HLA) that could be produced also by treatment of aldosterone in acid medium.

EXPERIMENTAL

Animals and materials

Rats. Male CHBB–Thom rats (200–250 g) were from the animal house at the FCEN.

Steroids. 18-Hydroxy-[1,2-^{3}H]corticosterone (sp. act. = 52 Ci/mmol) was obtained from The Radiochemical Center, Amersham, England. Aldosterone was purchased from Makor Chemicals, Jerusalem, Israel.

Buffers. pH 7.4 (Krebs–Ringer–bicarbonate); pH 4.8 (acetic acid acetate) and pH 3.3 (PO_4H_3–$PO_4H_2^-$) were prepared as in [1].

Scintillation counting and radioactivity scanning

A Packard 7201 radioactivity scanner was used to detect radioactive zones on chromatograms. A Tracor Analytic Mark III Model 6882 Liquid Scintillation System was used for counting the radioactivity in aliquots of each fraction (scintillation cocktail as in [1]).

NMR and mass spectra

^{1}H and ^{13}C NMR spectra were measured at 100 and 25.2 MHz respectively in a Varian XL-100-15 NMR spectrometer operating in the FT mode. Chemical shifts are in ppm downfield from TMS. Mass spectra were registered at 70 eV by direct inlet in a Varian Mat CH-7A mass spectrometer.

Isolation of HLA

Quartered rat adrenals were incubated with 20 pmol of 18-hydroxy-[1,2-^{3}H]corticosterone as previously described [1] with slight modifications. Briefly, incubations were carried out at pH 7.4, 4.8 or 3.3; after incubation, adrenals were separated from media and homogenized with 20% ethanol. Media and homogenates were then extracted with methylene chloride, the organic extracts were combined, evaporated under N_2 at 37°C and the dried residues were chromatographed in the Bush B5 system [2]. Radioscanning of paper strips evidenced 6 peaks (R_f 0.12, 0.35, 0.49, 0.61, 0.84 and 0.93). The radioactive material corresponding to a zone migrating to R_f 0.93 was eluted with methanol and the eluate was evaporated under N_2 at 37°C. The dried residue was then treated with 0.013 N aqueous ammonia at 30°C under N_2 in the dark for 24 h. Methanol (2 ml) was added and the solution evaporated (N_2, 37°C). The dried residue was again chromatographed in the Bush B5 system and paper strips were radioscanned. Two peaks could be evidenced at R_f 0.13 and 0.93. The material corresponding to the latter peak was eluted with methanol and the extracts were evaporated as above. The dried residue was saponified under N_2 with $KHCO_3$ (0.4% in methanol) for 8 h at 30°C [3].

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‡18-OH-B (M form) is a synonym of 4-pregnene-11β,18,21-triol-3,20-dione (mainly present as the 18–20 hemiketal); aldosterone is a synonym of 4-pregnene-18-oxo-11β,21-diol-3,20-dione (as a mixture of 11–18/18–20 ketal/hemiketal).

The products obtained were chromatographed in the Bush B5 system and two peaks were observed upon radioscanning at R_f 0.13 and 0.93. Finally the latter zone was submitted to the following chromatographic systems: Bush A (R_f 0.13); Cy-Di I [4] (R_f 0.78); TLC on silica gel: benzene–ethyl acetate 2:1 (R_f 0.40), hexane–acetone (5:4) (R_f 0.60). Upon acetylation [5] the eluted material yielded an acetate migrating in the Cy-Di I [4] paper chromatography system almost with the solvent front (R_f 0.96).

Preparation of HLA from aldosterone

Aldosterone (25 mg) was dissolved in hexadeuterodimethylsulfoxide (0.4 ml) and HCl vapours bubbled through the solution. Direct ^{13}C and ^{1}H NMR analysis of the reaction mixture indicated the presence of several forms in equilibrium with very broad signals. Hence the solution was diluted with water and extracted with methylene chloride. The residue obtained after evaporation of the solvent was mainly HLA (as determined by TLC) and the latter compound was isolated by preparative high performance chromatography on an Alltech R-Sil C18 HL 10 μm column (500 × 10 mm) with methanol–water (70:30) as eluent (Retention volume: 46 ml) at a flow rate of 4 ml/min.

RESULTS AND DISCUSSION

Table 1 shows yields of the highly lipophilic form of aldosterone (HLA) obtained in incubations of 18-HO-B with rat adrenals at different acidities. HLA could be isolated from these incubations, and was separated from the two other low polarity components that cochromatographed in the Bush B5 solvent system by treatment of the mixture: (a) with aqueous ammonia (24 h, 30°C) which converted one compound to 18-HO-B (M form), this compound could thus correspond to a low polarity derivative of 18-HO-B described by Aragonés *et al.* as RM 7.18 [6] which also reverts upon treatment with aqueous ammonia to 18-HO-B and (b) with $KHCO_3$ 0.4% in methanol (8 h, 30°C) according to Bush *et al.*[3] obtaining again 18-HO-B, probably due to hydrolysis of an 18-HO-B ester. It is interesting that lipoidal derivatives of some steroids such as corticosterone, pregnenolone and dehydroisoandrosterone have been described [7–10]. Table 1 also shows yields of the low polarity components migrating with HLA in the Bush B5 system, which were reverted to 18-HO-B prior to semiquantification. The only low polarity component remaining after the above treatment was HLA. The same compound could be produced by treatment of aldosterone with HCl 0.01 N (1 min, 25°C) or by bubbling HCl vapors through a solution of aldosterone in chloroform or in dimethylsulfoxide. The identity between the compound isolated from the incubations and the one prepared from aldosterone as indicated was established by comparison of their chromatographic properties on paper with several solvent systems, on Silica gel TLC plates, on RP-18 HPTLC plates and by reversed phase high performance liquid chromatography (RP-HPLC). Table 2 summarizes the chromatographic behavior of the above compounds. Also, the radioactive HLA from incubations with [1,2-^{3}H]18-HO-B was mixed with radioinert HLA (obtained from aldosterone plus HCl 0.01 N) and was crystallized to constant specific activity.

Table 1. Yields of HLA, saponifiable material (SM) and low polarity compound revertible to 18-OH-B with NH_3 (LPC) at various pHs

	% Products		
pH	HLA-^{3}H	SM-^{3}H	LPC-^{3}H
3.3	2.37	4.97	2.36
4.8	2.45	11.76	2.45
7.4	0.29	2.87	3.17

For the structure determination of HLA, aldosterone was dissolved in hexadeuterodimethylsulfoxide and HCl vapors were bubbled through the solution. Direct ^{13}C-NMR analysis of the solution indicated a rapid interconversion among several forms, presenting very broad signals for carbons 9, 11, 14, 15, 16, 18, 20 and 21 and single lines for carbons 4, 5, 7, 8 and 17. HLA was isolated by dilution with water and extraction with dichloromethane and was further purified by preparative RP–HPLC. The dimeric structure (I) was proposed for HLA based on the following spectroscopic evidences.

The ^{13}C-NMR spectra of aldosterone in Cl_3CD and in DMSO-d_6 (Table 3) are indicative of the presence

Table 2. Chromatographic behaviour of aldosterone and low polarity components

	R_f on paper chromatography			R_f on Silica gel TLC		R_f on RP-18 HPTLC	
Compound	Bush B5	CyDi I	Bush A	Hexane–acetone (5:4)	Benzene–ethyl acetate (2:1)	Methanol–water (7:3)	Retention volume in analytical HPLC*
Aldosterone	0.31	0.22	0	0.39			4.6 ml
HLA	0.93	0.78	0.13	0.60	0.40	0.31	16.2 ml
SM	0.93			0.71			22.3 ml
LPC	0.93			0.63			18.4 ml

*Column: μ Bondapak C-18 (300 × 3.9 mm) with methanol–water (70:30) as eluent at a flow rate of 1 ml/min.
(See Table 1 for abbreviations)

Table 3. NMR spectral data of aldosterone and HLA

	^{13}C-NMR (25.2 MHz)			^{1}H-NMR 100 MHz
Position	Aldosterone II*	Aldosterone III*	HLA I†	HLA I†
1	36.26	36.26	(39.00)	
2	35.12	35.12	36.10	
3	197.80	197.80	198.81	
4	123.26	123.17	124.24	5.72 (bs)
5	170.32	170.12	169.38	
6	33.18	33.18	33.71	
7	31.56	29.22	32.93	
8	30.75	31.96	32.03	
9	50.82	45.65	(48.69)	
10	37.97	37.29	(38.49)	
11	73.33	80.29	81.26	4.81 (bd) J = 6 Hz
12	39.70	38.41	40.33	
13	57.46	63.41	64.11	
14	51.95	48.11	(50.44)	
15	22.90	22.90	29.58	
16	24.42	25.46	25.91	
17	56.05	55.97	57.20	
18	95.21	106.74	(106.18)	5.21 (bs)
19	18.15	18.04	18.47	1.29 (s)
20	208.24	105.71	(105.64)	
21	67.82	66.43	66.89	3.49 (d); 3.98 (d) J = 13 Hz

*Solvent DMSO.d_6; †solvent $CDCl_3$.
Chemical shifts referred to TMS. Values in parentheses may be interchanged. bs: broad signal; bd: broad doublet; s: singlet; d: doublet.

of two isomeric forms II and III, probably in equilibrium, as proposed by Genard[11]. De Vries and De Jong[12] have reported the separation of forms II and III by HPLC and the partial conversion (possibly reversible) of II into III in the presence of NaOH 0.067 N in 85% ethanol. Our results indicate that the aldosterone used in our experiments may already be a mixture of these two isomeric forms. The ^{13}C-NMR data of HLA (Table 3) is similar to that of form III, although it presents significant differences for carbons 7, 9, 10, 11, 12, 14, 15 and 17; thus C-18 is acetalic and C-20 is hemiketalic as in III. The ^{1}H-NMR spectrum of HLA (Table 3) presents an AB quartet for the 21-hydroxymethylene group also consistent with a hemiketalic C-20; the broad singlet at 5.21 ppm and the broad doublet at 4.81 ppm corre-

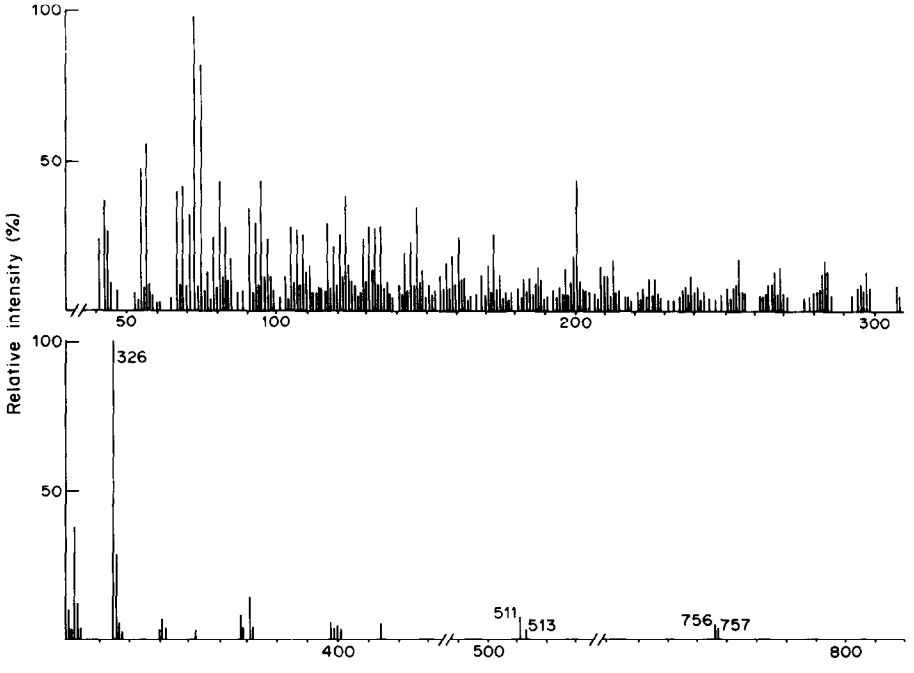

Fig. 1. Mass spectrum of HLA-tetratrimethylsilyl ether.

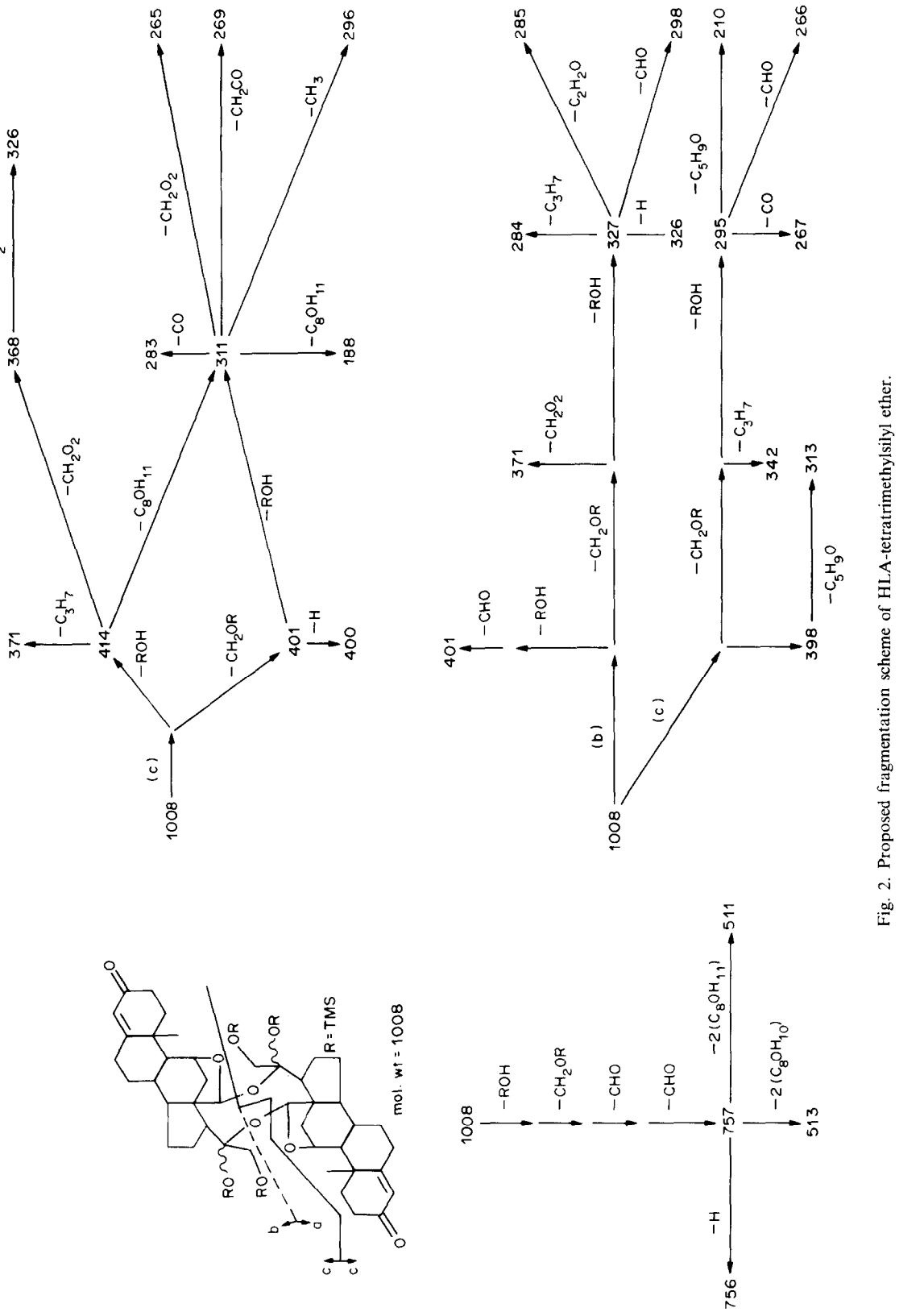

Fig. 2. Proposed fragmentation scheme of HLA-tetratrimethylsilyl ether.

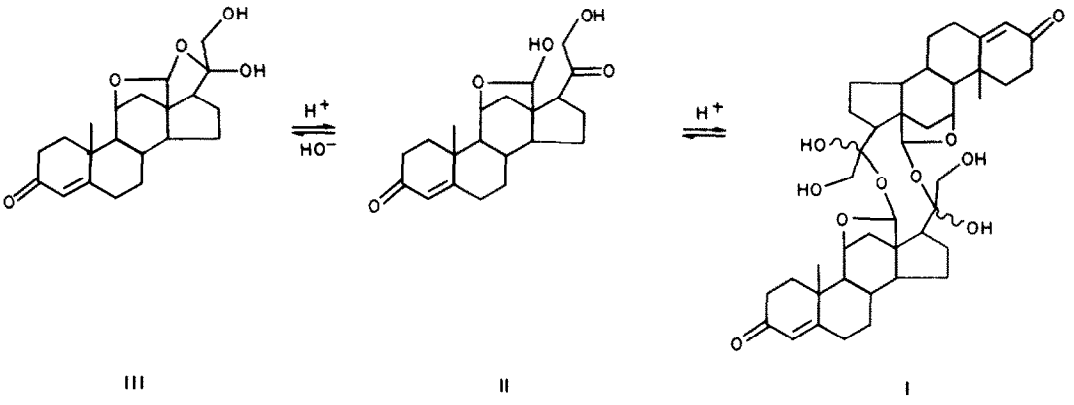

Fig. 3. Interconversion between the isomeric forms of aldosterone and HLA(I).

spond to H-18 and H-11 respectively. Considering the symmetry of the 2 aldosterone molecules in the dimer it can be assumed that the symmetrical carbons and protons are equivalent giving resonances at the same frequency.

The mass spectrum of underivatized HLA did not show a molecular ion. The heaviest ion observed had m/z 284 and may be ascribed to cleavage through bonds C-18–O and C-17–C-20 ($C_{19}H_{24}O_2$). Also observed was an ion m/z 256 ($C_{18}H_{24}O$: 284-CO). The mass spectrum obtained when HLA was sililated with BSTFA [12] is presented in Fig. 1. Although a molecular ion was not observed in this case either, the fragments at m/z 756/757 and 511/513 confirm the dimeric structure of the compound. Their proposed origin is summarized in Fig. 2, together with other relevant fragments observed.

The acid catalysed formation of I may be explained considering that: (a) if III is formed from II in basic conditions [12], this might be reversed by acid; (b) two molecules of II may experience an acid catalysed reaction to give I (Fig. 3). The low polarity of the dimeric structure I may be explained by assuming that the four hydroxyl groups of HLA are hidden inside the lipophilic structure formed by the steroid nuclei.

The existence of a dimer has also been postulated for 18-HO-B on the basis of its low retention volume on Sephadex LH 20 columns [13]. It should be mentioned that Harnik *et al.* obtained irreversibly during the hydrolysis of aldosterone diketal, aldosterone 3-ketal and several other less polar derivatives with defined structures differing from that of the dimer here reported [14]. On the other hand, Vecsei's group reported recently the isolation of an intramolecular acetal of aldosterone, 11,18-18,21-diepoxyaldosterone, with R_f 0.85 in the Bush B5 system under acidic conditions generally applied for aldosterone estimation in urine samples [15]. A minor product also isolated upon acid treatment of aldosterone in hexadeuterodimethylsulfoxide had a ^{1}H-NMR spectrum in accordance with this latter structure.

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