reduced pressure. The residue was chromatographed on a column containing 400 g of neutral alumina (Woelm) using increasing percentages of ethyl acetate in benzene for development. The fractions containing material absorbing at 283 nm were combined to give a yield of 2.7 g of 17β-hydroxyestra-4,6-diene-3,17-dione (**1c**) based on uv analysis. This material was purified further by preparative tlc using benzene–acetone (9:1, v/v). The $\Delta^{4,6}$ product and a less polar material were eluted. The mass spectrum of the latter showed highest mass peaks of equal intensity at *m/e* 298 and 300. The infrared spectrum was similar to that of the 3-methyl ether of estradiol-17β and glc analysis indicated the presence of two compounds of approximately equal amounts. However, these could not be separated on several thin layer and paper systems and the problem was not investigated further. It appeared that they were ethyl ethers of estradiol-17β and the corresponding Δ^5 compound.

Preparation of Catalyst.—A solution of 1 g of rhodium chloride trihydrate and 6 g of triphenylphosphine in 120 ml of ethanol was refluxed for 30 min.[20] Upon cooling, the precipitated tris(triphenylphosphine)rhodium(I) chloride was collected, washed with cold ethanol and ether, and then stored at 4° in a stoppered vial.

Preparation of 6,7-Labeled 17β-Hydroxyestr-4-en-3-one (1b). A. Tritium Labeling.—A solution of 27.2 and 14 mg of tris(triphenylphosphine)rhodium(I) chloride in 2 ml of dioxane was stirred under 1 mol equiv of tritium gas. There was an apparent 3.5-Ci uptake in 7 days. After removal of tritium gas and solvent, the residue was chromatographed on silver nitrate impregnated silica gel plates as detailed above using chloroform–methanol (98:2, v/v). A radioscan of a small sample chromatographed in a similar fashion showed major radioactive peaks at the origin and in the area corresponding in mobility to 17β-hydroxyestr-4-en-3-one (**1b**). Material in this zone was eluted to give 273 mCi (7.7% yield based on tritium uptake). A portion was diluted with 4-^{14}C-labeled and unlabeled testosterone and was crystallized from benzene–hexane to constant specific activity. This was diluted further with carrier and then was acetylated with 50% acetic anhydride in pyridine at room temperature for 3 hr. After tlc and crystallization, analysis for ^{3}H and ^{14}C again was carried out on weighed crystals. Another portion of the double-labeled testosterone was refluxed with 2% KOH in methanol–water (1:1, v/v), and was analyzed by scintillation counting after purification by tlc as described previously.[4] The results are in Table I.

B. Deuterium Labeling.—A dioxane solution containing 2.5 g of 17β-hydroxyestr-4,6-dien-3-one (**1c**) and 1.25 g of the rhodium catalyst was stirred in a deuterium atmosphere 16 hr at ambient temperature and pressure. The residue from evaporation was chromatographed on 400 g of silica gel using benzene–ethyl acetate mixtures. 17β-Hydroxyestr-4-en-3-one (**1b**) came off the column with a 9:1 mixture and was purified further by preparative plate chromatography in benzene–ethyl acetate (9:1 and then 8:2, v/v) to give a material which was homogeneous on gas chromatographic analysis (QF-1): mp 172–173°; λ_{max} 241 nm; mass spectrometric analysis of the molecular ion (d_0 = 274) d_0 (3%), d_1 (4%), d_2 (93%). Oxidation with Jones reagent[21] gave estr-4-ene-3,17-dione: nmr δ 0.94 (s, 3 H, 18-methyl), 5.89 (d, J = 1.7 Hz, 1 H, 4-H).

Preparation of 7-Labeled Estrenedione.—6,7-Labeled 17β-hydroxyestr-4-en-3-one (**1b**) was refluxed with base and was purified by tlc in benzene–ethyl acetate (3:1, v/v). Oxidation with Jones reagent[21] gave 7-labeled estr-4-ene-3,17-dione (**1a**). Material which had been tritiated was diluted with estr-4-ene-3,17-dione-4-^{14}C and was crystallized repeatedly. There was an insignificant change in the ^{3}H/^{14}C ratio. Similarly, the deuterated product, after dilution with carrier (1:1), isolation, and crystallization showed one peak on gas chromatography [d_0 (51%), d_1 (3%), d_2 (47%)]. Mass spectrometric analysis of the molecular ion showed only d_0 and d_1 species [d_0 (51%)].

Incubation with *B. malorum*.—Estrenedione-7-^3H,4-^{11}C (^{14}C sp act., 2700 dpm/mg; ^{3}H/^{14}C ratio, 28.6) was incubated with respiring cultures of *B. malorum* for 20 hr and the 7β-hydroxyestrenedione product was isolated as described[11] and the acetate derivative was crystallized from benzene–hexane three times. Loss of tritium as judged by decrease in the ^{3}H/^{14}C ratio was 98%. The product from the 7β-deuterated substrate [d_1 (34%)] was analyzed by mass spectrometry directly as the alcohol, since the highest *m/e* value for the acetate was 270 ($M^+ - 60$). There was no detectable amount of deuterium on mass spectrometric analysis of the 7β-hydroxy product after three crystallizations from ethyl acetate.

Registry No.—**1a,** 13209-45-5; **1a** (7-T, 4-^{14}C), 31031-84-8; **1b** (6-T, 4-^{14}C), 35140-96-6; **1b** (7-T, 4-^{14}C) 35140-97-7; **1b** (6-D), 35140-98-8; **1b** (7-D), 35140-99-9; **1c,** 14531-84-1.

Acknowledgment.—We thank Mssrs. J. Cronin and D. Quarton for obtaining the nmr and mass spectra and we are grateful to Dr. M. Gut for his advice on carrying out reductions with tritium gas and for making available his catalytic reduction facilities.

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The Use of Propionic Acid–Hydrochloric Acid Hydrolysis in Merrifield Solid-Phase Peptide Synthesis

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Merrifield solid-phase peptide synthesis[2-5] procedure is useful for the synthesis of peptides in high yield. However, several cases have been reported of incomplete couplings (for examples see ref 6–10). As work continues with the synthesis of larger peptides and proteins, the need for quick accurate analysis is becoming more important.

During the last 4 years we have been using anaerobic and aerobic propionic acid–hydrochloric acid (HCl) hydrolysis as an analytical tool. Our previous report[11] showed that blocked amino acids could be hydrolyzed easily from the resin used in Merrifield synthesis using this technique. This communication reports the results of 70 peptides hydrolyzed by these procedures, as compared to hydrogen fluoride–anisole cleavage from the resin, followed by constant boiling HCl hydrolysis.[12]

Table I gives the ratios, R, of moles of amino acids obtained from peptide resins hydrolyzed by 1:1 propionic acid–12 *N* HCl at 130° for 2 hr, and the moles of amino acids obtained from peptide resins treated with hydrogen fluoride–anisole[13,14] and then hydrolyzed by

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TABLE I

Amino acid	Side chain blocking group	Number of residues used[16]	$R_{aerobic}$[a]	95% confidence level[b]	R anaerobic[a]	95% confidence level[b]
Alanine		29	1.00	0.023	1.01	0.018
Arginine	Nitro	18	0.99	0.025	0.99	0.015
Aspartic	*O*-Benzyl	2	0.95	0.381	0.96	0.597
Cysteine	Methoxybenzyl	1	0.97		0.98	
Glutamic	*O*-Benzyl	17	0.99	0.015	1.01	0.013
Glycine		46	1.00	0.008	1.00	0.006
Histidine	Dinitrophenyl	7	0.81	0.076	0.98	0.047
Isoleucine		10	0.99	0.045	0.99	0.027
Leucine		14	1.00	0.019	0.99	0.011
Lysine	Carbobenzoxy	9	1.00	0.030	0.98	0.032
Methionine		3	0.94	0.133	0.98	0.120
Phenylalanine		19	1.01	0.017	1.00	0.021
Serine	*O*-Benzyl	54	0.73	0.018	0.85	0.014
Threonine	*O*-Benzyl	10	0.94	0.050	0.95	0.016
Tyrosine	*O*-Benzyl	8	0.80	0.061	0.90	0.045
Valine		13	0.99	0.024	0.99	0.009
Asparagine		15	0.97	0.051	1.01	0.041
Glutamine		15	1.00	0.017	1.00	0.013
Proline		3	0.93	0.013	0.97	0.022

[a] R is the average of the ratios of moles of amino acids obtained from resin peptides hydrolyzed by 1:1 propionic acid–12 *N* HCl at 130° for 2 hr to the moles of amino acids obtained from resin peptides by using hydrogen fluoride–anisole followed by HCl:H_2O hydrolysis at 100° for 24 hr. The ratios used for the calculation of R are normalized to the expected ratios in the synthetic peptide. No assumption about absolute yield of the peptide from the resin is used in the calculation. [b] The error limits for R were calculated using the Student's t distribution of $s = \sqrt{\Sigma(R - R_i)^2/n(n-1)}$ for $n - 1$ degrees of freedom and 95% confidence of the value R, where R is the mean value of the individual value R_i. These error limits, therefore, include errors in the single amino acid analyses,[16] as well as all other random errors in our experiments.

1:1 H_2O:12 *N* HCl at 100° for 24 hr. A wide variety of peptides were hydrolyzed in these experiments.[15] As would be expected, anaerobic propionic–HCl acid hydrolysis gives better results than aerobic hydrolysis. However, the aerobic procedure certainly can be used with excellent results. The only amino acids giving low recoveries by our procedure are serine, tryptophan, tyrosine, and threonine—all of which give low values with 6 *N* HCl hydrolysis. Tryptophan is, of course, completely destroyed by acid hydrolysis. The serine value seems to be quite temperature dependent.

The histidine values reported in Table I are rather uncertain. Several of the peptide resins examined contained incompletely coupled histidine. Some of the peptide molecules on these resins, therefore, contain histidine and some do not. It is known[10] that hydrogen fluoride–anisole will not cleave sterically hindered resin peptide molecules, whereas the propionic acid–HCl procedure cleaves all resin peptide molecules completely. The peptide molecules lacking histidine are expected to be those in the most sterically hindered positions on the resin and, therefore, the inaccuracy in the histidine values is understandable.

Hyrolysis of propionic acid–HCl at 130° facilitates quick, reliable analysis. The peptide doesn't require prior cleavage from the resin, and the preparation for amino acid analysis requires only 2 hr.

(15) Gly-Gln-Tyr-Ser-Trp-*Ile*-*Ile*-Asn-Gly-*Ile*-Glu-Trp-*Ala*-*Ile*-*Ala*-Asn-Asn-Met-Asp-Val; Asn-Ser-His-Gly-Thr-His-Val-Ala-Gly-Thr-Val-Ala-Ala-Leu-Asn-Asn-Ser-Ile-Gly; Ser-Met-Ala-Ser-Pro-His-Val-Ala-Gly-Ala-Ala-Ala-Leu-Ile-Leu-Ser-Lys-His-Pro; Asn-Trp-Thr-Asn-Thr-Gln-Val-Arg-Ser-Ser-Leu-Gln-Asn-Thr-Thr-Thr; Ser-Arg-Phe-Ser-Phe-Gly-Ala-Glu-Gly-Gln-Lys; Ser-Arg-Val-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys; Ser-Arg-Phe-Ala-Trp-Gly-Ala-Glu-Gly-Gln-Lys; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Ile-Lys; Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Ile; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Ile-Gly-Gln-Lys; Ser-Arg-Phe-Ser-Val-Gly-Ala-Glu-Gly-Gln-Lys; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln; Glu-Trp-Ala-Ile-Ala-Asn-Asn-Met-Asp-Val; Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Gly-Arg; Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gly-Gln-Arg; Phe-Ser-Trp-Ala-Ala-Glu-Gly-Gln-Arg; Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg; Phe-Ser-Trp-Gly-Ala-Glu-Gly-Glu-Arg; Act-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg; Phe-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg; Gly-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg; Ser-Thr-Gly-Ser-Ser-Ser-Thr-Val-Gly; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly; Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln; Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala; Thr-Ser-Ala-Ala-Ser-Ser-Ser-Asn; Arg-Ala-Ser-Phe-Ser-Ser-Val-Gly; Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg; Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg; Phe-Ser-Tyr-Ala-Glu-Gly-Gln-Arg; Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu; Phe-Ser-His-Ala-Glu-Gly-Gln-Arg; Trp-Gly-Ala-Glu-Gly-Gln-Arg; Ser-Arg-Phe-Ser-Trp-Gly-Ala; Pro-Gly-Asn-Lys-Tyr-Gly; Ala-Ala-Ser-Ser-Ser-Asn; Val-Glu-Gly-Leu-Tyr-Leu; Glu-Ala-Leu-Tyr-Leu-Val; Ser-Arg-Phe-Gly-Ser-Trp; Gly-Ala-Gln-His-Gly; Gly-Ala-Gln-Gly-Gly; Gly-Gly-Gln-Lys-Gly; Gly-Lys-Gln-Ile-Gly; Gly-Lys-Gln-Ala-Gly; Gly-Gly-Gln-Ser-Gly; Gly-Ser-Gln-Arg-Gly; Gly-Asp-Gln-Pro-Gly; Gly-Pro-Gln-Asp-Gly; Gly-Gln-Asn-Lys-Gly; Phe-Ser-Trp-Gly-Ala-Glu; Ser-Arg-Phe-Ser-Trp; Phe-Ser-Trp-Gly-Ala; Val-Glu-Gly-Leu-Tyr; Glu-Gly-Leu-Tyr-Leu; Asn-Gln-Ala-Ser-Phe; Gly-Gly-Leu-Tyr; Ala-Asp-Cys-Ser; Gly-Leu-Tyr; Leu-Gly-Glu; His-Gly; His-Glu; Pro-Glu; Tyr-His; His-Arg; Glu-His; Glu-Pro; Glu-Gly.

Experimental Section

The peptides were synthesized on chloromethylated copolystyrene crosslinked with 2% divinyl benzene resin by the procedure of Merrifield,[2-4] with occasional small modifications.[5] The resin was substituted with 0.2–0.5 mmol/g of the carboxyl terminal amino acid. The α amino groups of the amino acids were blocked by the *tert*-butoxycarboxyl groups, and the side chain groups were blocked as shown in Table I. One milliliter of propionic acid and 1 ml of 12 *N* HCl were placed in a small test tube with 1–3 μmol of the resin peptide, and the test tube was sealed. For anaerobic propionic–HCl acid hydrolysis, the acid mixture was frozen and thawed under vacuum three times, and then sealed under vacuum. The tube was later placed in either a heating block or a thermostated oil bath for 2 hr at 130°. The tubes were cooled and opened, and the samples were dried by rotary evaporation in 100-ml round-bottomed flasks at 40°. Amino acid analyses were then performed.[16] In separate experiments the same peptides were removed from the resin using anhydrous hydrogen fluoride and anisole.[13,14] After freeze drying, the peptides were hydrolyzed anaerobically with constant boiling HCl and analyzed. For aerobic hydrolysis the experimental procedure was the same, except that the tubes were sealed in air without exposure to vacuum.

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Registry No.—Propionic acid, 79-09-4; hydrochloric acid, 7647-01-0.

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The Origin of the $[M - 56]\cdot^+$ Ion in the Mass Spectra of Trimethylsilyl Ethers of Dehydroepiandrosterone and Related Compounds

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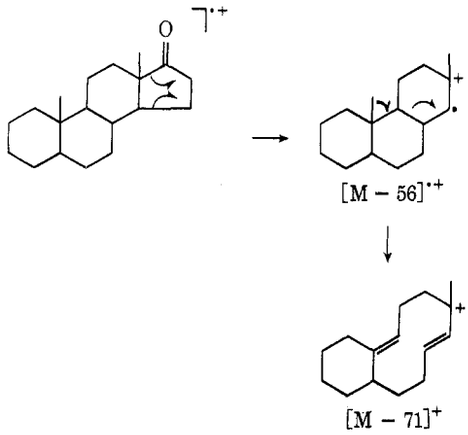
The mass spectra of many 16- and 17-keto steroids contain ions $[M - 56]\cdot^+$, the formation of which has been ascribed to cleavages of the bonds C-13/17 and C-14/15.[1] These ions are often accompanied by ions $[M - 71]^+$ formed by subsequent loss of a methyl radical[2] (Scheme I). During a survey of the mass

SCHEME I

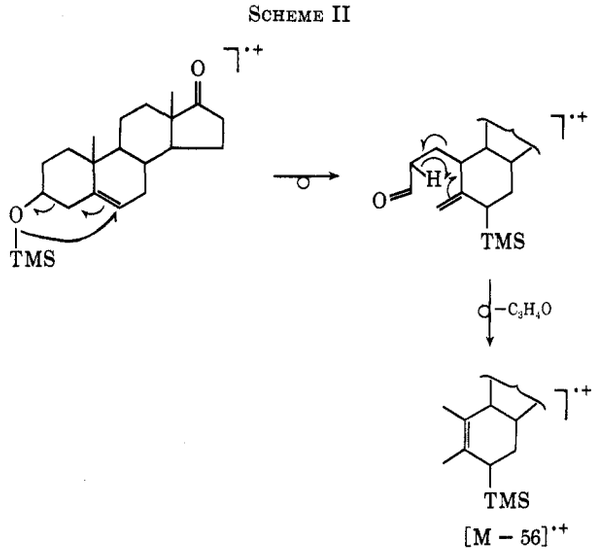


spectra of trimethylsilyl (TMS) ethers of a number of Δ^5-3β-hydroxy steroids it was found that $[M - 56]\cdot^+$ ions, unaccompanied by $[M - 71]^+$ ions, were present in the spectra of 16 and 17 ketones. It has been demonstrated that these $[M - 56]\cdot^+$ ions are formed by electron-impact-induced rearrangement, and not by D-ring cleavage.

The first indication of a duality of mechanisms for the formation of such ions was the presence of $[M - 56]\cdot^+$ ions in the spectra of the TMS ethers of *15,15,17,17-d_4*-3β-hydroxyandrost-5-en-16-one, *16,16-d_2*-3β-hydroxyandrost-5-en-17-one, and *9,12,12,16,16-d_5*-3β-hydroxyandrost-5-ene-11,17-dione.[3] When the 17-oxo group of the TMS ether of 3β-hydroxyandrost-5-en-17-one (dehydroepiandrostereone, DHEA) was selectively replaced[4] by ^{18}O, this atom was found to be retained in the $[M - 56]\cdot^+$ ion. All nine deuterium atoms of the d_9-TMS ether[5] of DHEA were also retained in the $[M - 56]\cdot^+$ ion.

High resolution mass measurement, carried out on the spectrum of the TMS ether of DHEA, showed that the particle eliminated had the composition C_3H_4O (found for ion of nominal *m/e* 304, 304.2198; calcd for $C_{19}H_{32}OSi$, 304.2222). The oxygen atom must, therefore, originate from the 3 position, and it seems likely that the $[M - 56]\cdot^+$ ions of these steroids are formed by a mechanism similar to that proposed for the formation of the $[M - 129]^+$ ion, but with initial transfer of the TMS group. This may proceed *via* a double (silyl and conventional) McLafferty-type rearrangement, as in Scheme II. Because of the relatively large separation

SCHEME II



of C-3 and C-6, the silyl rearrangement is presumed to take place in a stepwise manner.

It should be noted that the TMS ethers of the saturated steroid 3β-hydroxy-5α-androstan-17-one and its 16,16-d_2 analog give rise, respectively, to ions $[M - 56]\cdot^+$ and $[M - 58]\cdot^+$, indicating that such ions are formed by D-ring cleavage as illustrated in Scheme I.[6]

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