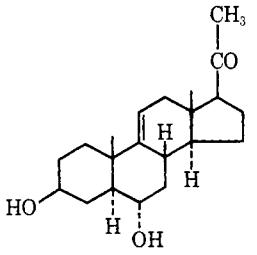
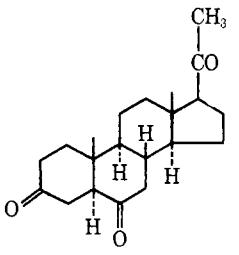
43, and strong fragments generated by the loss of water and methyl groups were observed at *m/e* 314, 299, 296, and 281. The most important are the prominent peaks at *m/e* 230, 229, and 211, which correspond to D-ring fission products of a pregnan-20-one derivative[4] accompanied by dehydration, and, if it is the case, it also implies that two oxygen functions are located somewhere on the A, B, or C ring. The nmr spectrum[5] showed two angular methyl signals at τ 9.47 and 9.05, and a methyl proton of a methyl ketone (τ 7.89). The presence of a methyl ketone supports the information obtained from the mass spectrum. There are also signals for one olefinic proton, τ 4.63 (unresolved triplet), and two protons at *ca.* τ 6.4, indicating the presence of a trisubstituted double bond and two secondary hydroxyl groups. Application of Zürcher's table,[6] on the assumption that **2** has a pregnane skeleton, led to two structures, *i.e.*, 3β,6α-dihydroxy-$\Delta^{9(11)}$-5α-pregnan-20-one and 3β,11α-dihydroxy-Δ^{7}-5α-pregnen-20-one as compounds expected to have the closest chemical shifts for the angular methyl groups. The shielded nature of the 18-methyl group can be explained only by locating the trisubstituted bond at the 9(11) or 7 position. Any allylic alcohol type structures were excluded considering a great stability of the compound under a strong acidic conditions.

Final confirmation of the structure was accomplished by the following correlational work.

Hydrogenation of **2** with Adams' catalyst in acetic acid readily reduced the double bond with partial reduction of the keto group. Usually under the conditions used, Δ^{7} double bonds cannot be reduced, but migrate to the $\Delta^{8(14)}$ position.[7] The oxidation of the reduction products with Jones' reagent afforded a saturated triketone, **3**, mp 233–235° (M^+, *m/e* 330;



2



3

quantitative yield from **2**), which was found identical with authentic 5α-pregnane-3,6,20-trione[8] by mixture melting point, tlc, and comparison of the ir and mass spectra. The stereochemistries of 3- and 6-hydroxyl groups were assigned as β and α, respectively, because the broad nature of the 3- and 6-methine proton signals (the width at half-height, *ca.* 20 Hz each) indicates that both protons are axially oriented; accordingly the hydroxyl groups are equatorial. The chemical shift of the 19-methyl group (τ 9.05) also rules out the β configuration of the 6-hydroxyl group which would be in the relation of 1,3 diaxial to the 19-methyl group and would cause a big downfield shift (about 0.2 ppm). Thus, the structure of **2** was established as 3β,6α-dihydroxy-5α-pregn-9(11)-en-20-one.

Pregnane derivatives have never been reported in such lower animals as Echinoderms, and even in the whole invertebrate very few have been reported. The origin of **2** and its relationship to the other aglycons are currently under study.

Acknowledgments. This work was supported by the Sea Grant Program, University of Rhode Island. The author thanks Dr. H. W. Youngken and Dr. L. R. Worthen for encouragement throughout this work.

Yuzuru Shimizu

Department of Pharmacognosy, College of Pharmacy
University of Rhode Island
Kingston, Rhode Island 02881

Received February 22, 1972

(4) L. Tokes, R. T. LaLonde, and C. Djerassi, *J. Org. Chem.*, **32**, 1020 (1967), and most recently S. Popov, C. Eadon, and C. Djerassi, *ibid.*, **37**, 155 (1972).

(5) The nmr spectrum was taken in $CDCl_3$ with TMS as standard.

(6) R. F. Zürcher, *Helv. Chim. Acta*, **44**, 1380 (1961); (b) *ibid.*, **46**, 2054 (1963).

(7) H. Wieland and W. Benend, *Justus Liebigs Ann. Chem.*, **554**, 1 (1943).

(8) (a) R. E. Marker, E. M. Jones, D. L. Turner, and E. Rohrmann, *J. Amer. Chem. Soc.*, **62**, 3006 (1940); (b) S. Lieberman, K. Dobriner, B. R. Hill, L. F. Fieser, and C. P. Rhoads, *J. Biol. Chem.*, **72**, 263 (1948).

The Fluorogenic Ninhydrin Reaction. Structure of the Fluorescent Principle

Sir:

The reaction of phenylalanine with ninhydrin in the presence of peptides generates fluorescent materials.[1] This discovery forms the basis of an efficient assay for serum phenylalanine which is employed in the diagnosis of phenylketonuria.[2] Recently, it has been shown[3] that the condensation of primary amines (including peptides) with ninhydrin and phenylacetaldehyde, *i.e.*, the oxidative decarboxylation product of phenylalanine, yields highly fluorescent, ternary products. This sensitive reaction is utilized in a novel automated fluorometric procedure for the assay of primary amines which is particularly valuable for the detection of peptides in nanomole quantities.[4] We now wish to report that the major fluorescent principle of these reactions possesses the general structure **1** (Scheme I).

This result emerged from the investigation of a model reaction with ninhydrin, phenylacetaldehyde, and ethylamine.[3] Heating of equimolar amounts of these components in aqueous methanol afforded three interrelated products, two of which strongly fluoresce upon irradiation. The major fluorescent component (70% of the isolated products) crystallized upon concentration of the chloroform extract of the acidified reaction mixture. A minor fluorophor (22%) and a nonfluorescent compound (8%) were separated from the mother liquor by preparative thin layer chromatography.

Structure **2** was established for the major fluorophor [$C_{19}H_{17}NO_4$;[5] mp 247°; uv max (CH_3OH) 275 (ϵ 18,900) and 386 nm (6000); ir (KBr) 1680 and 1620 cm^{-1}; nmr (DMSO-d_6) δ 9.07 (s, NEtC*H*=); mass spectrum *m/e* 305 (M − 18, 100%); fluorescence spectrum, excitation max 288 and 395 nm, emission max 485

(1) I. P. Lowe, E. Robins, and G. S. Eyerman, *J. Neurochem.*, **3**, 8 (1958).

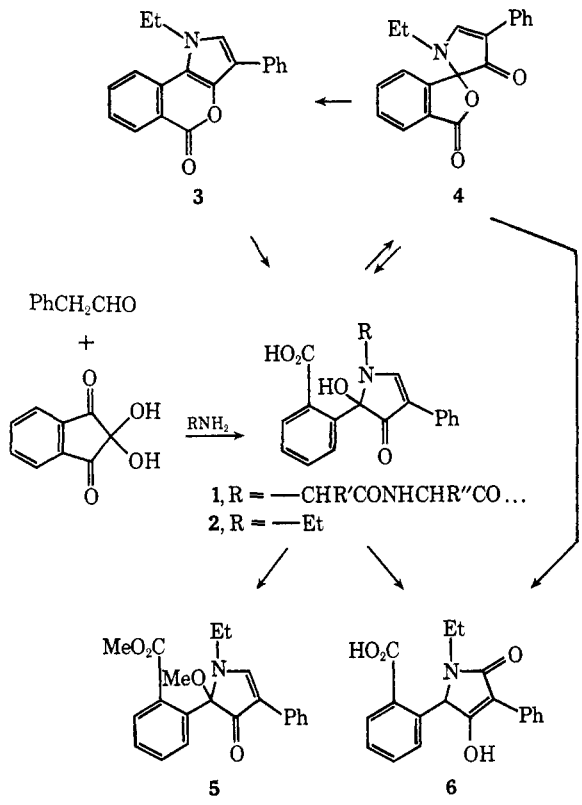
(2) (a) M. W. McCaman and E. Robins, *J. Lab. Clin. Med.*, **59**, 885 (1962); (b) P. W. K. Wong, M. E. O'Flynn, and T. Inouye, *Clin. Chem.*, **10**, 1098 (1964).

(3) K. Samejima, W. Dairman, and S. Udenfriend, *Anal. Biochem.*, **42**, 222 (1971).

(4) K. Samejima, W. Dairman, J. Stone, and S. Udenfriend, *ibid.*, **42**, 237 (1971).

(5) All new compounds gave satisfactory elemental analyses. Melting points are uncorrected.

Scheme I



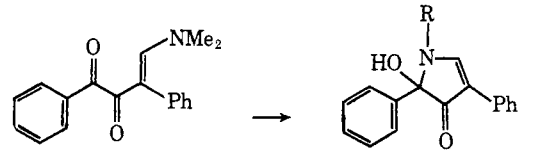
nm)]. The minor fluorophor possesses structure **3** [$C_{19}H_{15}NO_2$; mp 138°; uv max (CH_3OH) 240 (ϵ 29,750), 291 (19,800), and 372 nm (5800); ir ($CHCl_3$) 1710 cm^{-1}; nmr ($CDCl_3$) δ 6.97 (s, NEtC*H*=); mass spectrum *m/e* 289 (100%); fluorescence spectrum, excitation max 302 and 377 nm, emission max 480 nm]. The nonfluorescent product has structure **4** [$C_{19}H_{15}NO_3$; mp 184°; uv max (CH_3OH) 267 (ϵ 20,100) and 386 nm (5900); ir ($CHCl_3$) 1690 and 1790 cm^{-1}; nmr δ 8.48 in $CDCl_3$, 9.37 in DMSO-d_6 (s, NEtC*H*=); mass spectrum *m/e* 305 (100%)].

Since the fluorescence properties of **2** closely resemble those of the peptide-derived fluorophors,[3] it is concluded that their structure should be expressed by formula **1** (in which R signifies a peptide residue).

The assignment of structures **2**, **3**, and **4** relies to a significant extent upon the interpretation of the chemical transformations which are outlined in Scheme I. The minor fluorophor **3** evidently arises from the components of the reaction as a ternary condensation product, while formation of the major fluorophor **2** and the nonfluorescent congener **4** requires an additional, oxidative reaction step. Accordingly, **3** was readily converted to **2** upon heating in methanolic potassium hydroxide in the presence of air. The same transformation can also be effected efficiently with ninhydrin in weakly alkaline solution. This finding suggests that compounds of type **3** may actually be the precursors of the observed fluorophors **1** under assay conditions where a large excess of ninhydrin is utilized.[4] On the other hand, **4** was smoothly reduced to **3** by hydrogenation over Pd/C in glacial acetic acid. Brief heating of the hydroxy acid **2** in isopropyl alcohol afforded quantitatively the lactone **4**. The reversal of this cyclization, though not without side reactions, was effected with aqueous sodium bicarbonate. Alkylation of **2** in hexamethylphosphoramide with potassium *tert*-butoxide and dimethyl sulfate yielded the fluorescent dimethyl derivative **5** [mp 124°; uv max (CH_3OH) 273 (ϵ 16,300) and 390 nm (4900); ir ($CHCl_3$) 1735 and 1690 cm^{-1}]. Treatment of **2** with hydrochloric acid in acetone gave the tetramic acid **6** [mp 249°; uv max (CH_3OH) 236 (ϵ 20,700), 273 (10,000), and 279 nm (10,100); nmr (DMSO-d_6) δ 6.24 (s, benzylic proton), 3.55 and 2.74 (AB of quartets, CH_3CH_2N). The same intramolecular disproportionation occurred upon exposure of **4** to acid or base. An X-ray crystallographic analysis secured the structure of **6** [monoclinic crystals, space group $P2_1/c$, with a = 8.530, b = 15.166, and c = 12.841 Å, β = 95.75°, Z = 4, d_{obsd} = 1.29 g cm^{-3}]. The structure was solved by a straightforward application of the symbolic addition method[6] and was refined by least squares. The final agreement factor R is 4.4% (all atoms except hydrogen anisotropic).

In order to further corroborate structure **2**, a series of simplified analogs was synthesized (Scheme II).

Scheme II



Formylation of 1,3-diphenyl-1,2-propanedione[7] with excess *N,N*-dimethylformamide dimethyl acetal furnished the dimethylaminomethylene derivative **7** [mp 108°; uv max (CH_3OH) 250 (ϵ 12,200) and 300 nm (13,300)]. This substance, upon reaction with ethylamine in ethanol, was readily converted to the fluorescing pyrrolinone **8a** [mp 138°; uv max (CH_3OH) 281 (ϵ 18,700) and 385 nm (6080); nmr δ 8.03 ($CDCl_3$), 9.06 (DMSO-d_6) (s, NEtC*H*=)]. Analogously, *p*-bromobenzylamine afforded the fluorophor **8b** [hemimethanolate; mp 127–128°; uv max (CH_3OH) 279 (ϵ 16,700) and 385 nm (6580)]. Solid evidence for the cyclic nature of these compounds was provided by the nmr spectrum ($CDCl_3$) of **8b**, which shows an AB pattern for the benzyl protons (δ 4.30 and 4.13; J = 15 Hz). The observed nonequivalence of these protons indicates their proximity to a center of chirality, a requirement which is met by the proposed cyclic structure. The structure **8b** was further confirmed by an X-ray crystallographic analysis. Crystals of **8b** are triclinic, space group $P\bar{1}$, with four molecules (plus two molecules of methanol) in a unit cell with dimensions a = 10.519, b = 12.444, and c = 16.175 Å, α = 76.00, β = 83.72, γ = 88.58° (d_{obsd} = 1.41 g cm^{-1}). The structure was solved by the heavy atom method and was refined by least squares. All hydrogens (except those of methanol) were located from a difference synthesis. The final R is 4.6% (all atoms except hydrogen anisotropic).

Since the spectral properties of the major fluorophor **2** are in perfect agreement with those of the analogs **8a** and **8b**, all of the proposed structures are secured.

The utilization of these results in the design and syn-

(6) I. L. Karle and J. Karle, *Acta Crystallogr.*, **16**, 969 (1963).
(7) O. Widman, *Chem. Ber.*, **49**, 477 (1916).

thesis of a novel reagent, which will supersede the fluorogenic ninhydrin reaction for the assay of primary amines, shall be the subject of a forthcoming report.

Acknowledgments. We thank Dr. S. Udenfriend for drawing our attention to this problem and Drs. W. Dairman and K. Samejima for providing their results prior to publication. We also express our gratitude to Professor G. Büchi for stimulating discussions.

M. Weigele,* J. F. Blount, J. P. Tengi
R. C. Czajkowski, W. Leimgruber
Chemical Research Department, Hoffmann-La Roche Inc.
Nutley, New Jersey 07110
Received February 18, 1972

Additions and Corrections

The Kinetics, Isotope Rate Effect, and Mechanism of Dehydrobromination of *cis*-1,2-Dibromoethylene with Triethylamine in Dimethylformamide [*J. Amer. Chem. Soc.*, **91**, 468 (1969)]. By W. K. KWOK, W. G. LEE, and SIDNEY I. MILLER,* Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616.

Professor J. M. Lehn pointed out to us that the molecule we took to have the structure **5M**, $BrCH{=}CHN(CH_3)_3{}^+Br^-$, was really $H_2C{=}CBrN(CH_3)_3{}^+Br^-$. The former assignment of Bode's was corrected by F. Klages and E. Drerup, *Liebigs Ann.*, **547**, 65 (1941), and confirmed by M. Ohtsura, K. Tori, J. M. Lehn, and R. Seher, *J. Amer. Chem. Soc.*, **91**, 1187 (1969). The closest analog to the Bode structure or **5E** that we are aware of is $BrCH{=}CHN(C_2H_5)_2$, an unstable liquid [R. Tanaka and S. I. Miller, *J. Org. Chem.*, **36**, 3856 (1971)]. The argument against the SN2 process has been weakened, but the remaining evidence still favors the $(E1cb)_{ip}$ process.

k_{-4} (rather than k_4) should appear in the denominator of eq 12.

Book Reviews

Carbohydrate Chemistry. Volume 4. By J. S. BRIMACOMBE (University of Dundee). The Chemical Society, London. 1971. ii + 275 pp. £4.00.

The fourth volume of this Specialist Periodical Reports covers the literature published in 1970 and is arranged in two parts: (1) the chemistry of mono-, di-, and trisaccharides and their derivatives; (2) the structure and physical and biological properties of carbohydrate macromolecules.

Although the first part might be of greater usefulness for the carbohydrate chemist and the chemist of natural products, the detailed and well-organized list of contents should make it extremely valuable for teachers, analytical chemists, pharmacologists, and all those scientists interested in correlating chemical structure and biological or physical properties of carbohydrates. The coverage of the literature is excellent, and the content of the papers listed is summarized in an effective manner. Schemes, formulas, and diagrammatic representations are widely used to facilitate comprehension. Chapters 22–26 inclusive afford an excellent review of the recent analytical development in the field.

Although the emphasis of the volume is defined to be "throughout on chemical rather than biochemical aspects," the second part (due to Dr. J. F. Kennedy, University of Birmingham) should be of particular interest for the biochemists who might have missed some of the recent contributions. Again, the coverage of the topics is superb and their sequence so well organized that seldom do the reporters have to break the numerical sequence of their references in order to go back to contributions already mentioned. Chapters 2 to 5 inclusive (covering Glycoproteins, Glycopeptides, and Animal Polysaccharides; Enzymes Either Active on Carbohydrates or Containing Them; Glycolipids and Gangliosides; and Chemical Synthesis and Modification of Polysaccharides, Glycoproteins, Enzymes and Their Use) should be valuable not only to biochemists but also to clinical investigators interested in inborn errors of metabolism and to students who might wish to search for or to review some of the salient contributions made to the field in 1970.

Completed in August 1971, the volume was published the following November. Although the accumulating literature in this specialistic field made it look already like a labor of Sisyphus, the knowledge that this volume will be of help to a large cross section of chemists, scientists, and students should repay the reporters of their excellent efforts.

Nicola Di Ferrante, *Baylor College of Medicine*

Spectroscopic Properties of Inorganic and Organometallic Compounds. Volume 4. Senior Reporter: N. N. GREENWOOD (University of Newcastle upon Tyne). The Chemical Society, London. 1971. xviii + 604 pp. £10.00.

This volume is the fourth in this annual review series published as a Specialist Periodical Report by The Chemical Society, London. It surveys the literature published during 1970 and is divided into eight chapters: "Nuclear Magnetic Resonance Spectroscopy" by J. R. Blackborow and K. D. Crosbie; "Nuclear Quadrupole Resonance Spectra" by J. H. Carpenter; "Microwave Spectroscopy" by J. H. Carpenter; "Vibrational Spectra: General Introduction and Definitive Spectra" by B. P. Straughan; "Characteristic Vibrational Frequencies of Compounds containing Main-group Elements" by B. E. Prater; "Vibrational Spectra of Transition Element Compounds" by B. E. Prater; "Vibrational Spectra of Some Co-ordinated Ligands" by M. Kilner; "Mössbauer Spectroscopy" by R. Greatrex and N. N. Greenwood. This arrangement differs from earlier volumes in which esr, electronic spectra, and the magnetic properties of ionic solids and coordination compounds were included. A new series on these will be forthcoming.

The coverage in these chapters is quite thorough and indicates the prodigious effort which must have been expended by each reporter. That a review covering the 1970 literature could be written and published by October 1971 is indeed commendable.

One wonders, however, about the ultimate fate of annual review-type publications. They tend to discuss individual papers in such a succinct manner that the reader really finds out nothing about