with 100 ml of methanol and were recovered by evaporation of the solvent. These metabolites were presumed to be conjugates of hydroxylated compounds since they were readily hydrolyzable to less polar entities by incubation with a mixture of β-glucuronidase and aryl sulfatase (Glusulase, Endo products) at pH 5.5. That they were hydroxylated materials was confirmed as follows.

The hydrolyzed metabolites were recovered by extraction into CH_2Cl_2 and were examined by combined gas chromatography–mass spectrometry (Model LKB 9000) using an 8-ft column of 1% W-98 silicone gum rubber on Gas Chromosorb Q at 165°. Under these conditions GC peaks were seen with retention times of 9 and 11 min. The metabolite with the longer retention time had a molecular ion of 293 and a fragmentation pattern consistent with structure **3b**, *i.e.*, hydroxylated **3a**.

Conversion of **3b** to **3c** ($M^+ = 307$) by treatment with diazomethane confirmed the presence of a phenolic hydroxyl. Reaction of **3b** with acetic anhydride yielded **3d** ($M^+ = 335$). The fragmentation patterns of **3b**, **3c**, and **3d** were all consistent with the assigned structure.

Similarly the metabolite with the shorter retention time was found to have the structure **4b**. Reaction with diazomethane converted **4b** to **4c** while **4d** was formed by reaction with acetic anhydride. The mass fragmentation patterns of **4b** ($M^+ = 279$), **4c** ($M^+ = 293$), and **4d** ($M^+ = 321$) were all consistent with the structures assigned.

Enzymatic hydroxylation of an aromatic ring is a well-known pathway in the metabolism of exogenous substances in mammals (*cf.* Daly[5] for a recent review of aromatic hydroxylation and the role of arene oxide intermediates). Para hydroxylation is usually observed in the case of monosubstituted benzene rings. For example, para hydroxylation is the major pathway in the metabolism of diphenylhydantoin,[6] although meta hydroxylation has been observed as a minor pathway.[7] It is likely that metabolites **3b** and **4b** are *p*-hydroxy compounds, but this must be established by synthesis or additional physical studies. A significant point, however, is that although aromatic hydroxylation is an important pathway it has not as yet been reported as a route of metabolism of synthetic opiates.[8]

In order to obtain further information about **3b** and **4b**, the fate of radiocarbon-labeled methadone was studied in rats. Both **3b** and **4b** were found to be excreted as radioactive conjugates (either glucuronides or sulfates) in the bile of rats receiving methadone-*2*-^{14}C. Further confirmation of the nature of **3b** and **4b** came from the observation that the administration of **3a** to rats resulted in the excretion of conjugates of **3b** and **4b** into bile. When **4a** was administered only conjugated **4b** was detected.

In addition to the new metabolites, **3b** and **4b**, a further metabolite was found to be directly extractable from acidified human or rat urine. The chromatographic behavior and mass fragmentation pattern of this metabolite were identical with those of a known sample of 4-dimethylamino-2,2-diphenylpentanoic acid (**5**). This acid would be expected to arise *via* the oxidative removal of C-1 and -2 from methadone. The observation that 1–5% of a dose of methadone-*2*-^{14}C in the rat is expired as radiocarbon dioxide is consistent with this suggestion.

The formation of the carboxylic acid metabolite **5** has some precedent in the metabolic conversion of acylbenzenes to benzoic acid.[9] However, the usual route of metabolism of ketones is *via* enzymatic reduction,[10] a reaction which may be suppressed in methadone metabolism because of the highly hindered nature of this ketone.

The quantitative importance of these new metabolites has not as yet been completely established. However, their relative abundance in the urine of a typical methadone maintenance subject appears to be: **3a**, **4b** > **1** > **3b** > **4a**, **5**. In addition to further studies on metabolite quantitation we are also directing our attention to a resolution of the isomer problem represented by structures **3b** and **4b**.

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(7) A. J. Atkinson, J. MacGee, J. Strong, D. Garteiz, and T. E. Gaffney, *Biochem. Pharmacol.*, **19**, 2483 (1970).
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Characterization of an Acid Hydrolysis Product of Starfish Toxins as a 5α-Pregnane Derivative

Sir:

Starfish contain toxins which are cytotoxic, hemolytic, antiviral, and repellent to other marine animals.[1] The author has recently reported the purification of the toxins from three species of starfish.[2]

A toxin, ASP-II (**1**), from crown of thorns, *Acanthaster planci*,[3] is of glycosidic nature, and has a sulfate group and a molecular weight of about 1500 (*Anal.* Found: S, 2.08%). Upon hydrolysis with 2 *N* H_2SO_4, **1** gave sugars (quinovose and fucose in about a 2:1 ratio) and the aglycone fraction as a mixture of closely resembled compounds, from which, after repetitions of chromatography, a pure crystalline compound **2** was obtained. **2** constitutes about one-third of the aglycone fraction, and it was also isolated from the hydrolysate of toxins of *Asterias forbesi*, an Atlantic species.[3]

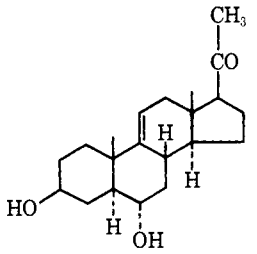
The compound **2**, long needles from methanol–water, mp 193–196°, $[\alpha]^{23}D$ +98.4° (methanol), was given the molecular formula $C_{21}H_{32}O_3$ on the basis of the mass spectrum and elemental analysis (M^+, *m/e* 332. *Anal.* Found: C, 75.62; H, 9.50%). The ir spectrum showed the presence of hydroxyl groups (3400 cm^{-1}) and a carbonyl absorption (1700 cm^{-1}). The absence of a conjugated system in **2** is evident from the lack of strong absorptions in the uv spectrum. In the mass spectrum, the base peak is acetylium ion, *m/e*

(1) B. W. Halstead, "Poisonous and Venomous Marine Animals of the World," Vol. 1, U. S. Government Printing Office, Washington, D. C., 1965, p 537, and also see the references cited in ref 2.
(2) Y. Shimizu, *Experientia*, **27**, 1188 (1971).
(3) *Acanthaster planci* was collected in Hawaii. *Asterias forbesi* was collected locally in May 1971.

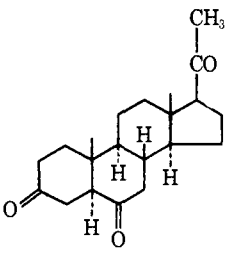
43, and strong fragments generated by the loss of water and methyl groups were observed at *m/e* 314, 299, 296, and 281. The most important are the prominent peaks at *m/e* 230, 229, and 211, which correspond to D-ring fission products of a pregnan-20-one derivative[4] accompanied by dehydration, and, 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



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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.

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

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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

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