Anal. Calcd. for $C_{11}H_9NO_2$: C, 70.58; H, 4.85; N, 7.48, mol. wt., 187. Found: C, 70.73; H, 4.96; N, 7.43. mol. wt., (Rast's method) 175.6.

The residual solid which was insoluble in petroleum ether (b.p. 70–80°) was repeatedly crystallized from benzene to give Δ^2-pyrazoline (III) in colorless crystals (0.03 g.), m.p. 212° with slight evolution of gases (brown melt). When III was heated in a vacuum at 200°, it sublimed unchanged.

Anal. Calcd. for $C_{11}H_9N_3O_2$: C, 61.39; H, 4.19; N, 19.53. Found: C, 61.80; H, 4.55; N, 20.25.

When the above-mentioned thermal decomposition was carried out in a vacuum at 130° for 0.5 hr. no remarkable change was observed, and the starting material was recovered unchanged (melting point and mixture melting point).

When the decomposition was carried out under vacuum for 0.5 hr. at 180°, the following products were isolated from 0.5 g. of the starting pyrazoline derivative: (a) 0.14 g. of crystalline *N*-phenylitaconimide (m.p. 98°), (b) 0.03 g. of the Δ^2-pyrazoline (m.p. 212°), and (c) 0.5 g. of the unchanged starting material (m.p. 178°); the cyclopropane derivative (V) (m.p. 125°) could not be isolated.

Preparation of N-phenylitaconimide. A mixture of itaconic acid monoanilide[13] (20 g.), fused sodium acetate (2 g.), and excess of acetic anhydride (30 ml.) was heated at 80–90° for 1 hr. The reaction mixture was then poured portionwise with stirring into ice-cooled water. The precipitated solid product was filtered off, then washed with water, and crystallized first from benzene–petroleum ether (b.p. 40–60°) to give *N*-phenylitaconimide in yellow needles, m.p. 110° (lemon yellow melt).

Anal. Calcd. for $C_{11}H_9NO_2$: C, 70.58; H, 4.85; N, 7.48. Found: C, 70.61; H, 4.68; N, 7.68.

When this product was heated for 1 hr. just above its melting point, and the melt was triturated with hot petroleum ether (b.p. 40–60°), the mother liquor gave on concentration the starting material (melting point and mixture melting point).

Preparation of cyclopropane-2,3-(N-phenyl)dicarboximide. It was prepared according to Perkin *et al.*,[2] the melt in the last step was extracted with petroleum ether (b.p. 60–80°), and then the extract was concentrated and cooled, whereby colorless needles separated. By repeated crystallization from the same solvent, it was found that the melting point rose to 125°; Perkin *et al.*[2] gave a m.p. of 98°.

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Microbiological Transformation of Steroids. IX. The Transformation of Reichstein's Compound S by *Scenedesmus sp.* Diketopiperazine Metabolites from *Scenedesmus sp.*

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Species of algae generally hold the unique position found in higher plants of being able to synthesize their own protoplasm from carbon dioxide, sunlight and inorganic nitrate. Most physiological studies with algae have been concerned with this autotrophic method of growth.

We, on the other hand, were particularly interested in determining the metabolic capabilities of algae insofar as they could be used to transform steroids. Our search for conditions for rapid cell proliferation led us into a study of the area of heterotrophic nutrition. Several species were found which grew exceptionally rapidly in a heterotrophic medium containing carbohydrate and a complex organic nitrogen source. These particular algae grew equally well in shake flasks and in aerated and agitated laboratory fermentors of ten liters capacity. Since light enhanced growth in the heterotrophic medium, one might consider that these species of algae spanned both autotrophic and heterotrophic conditions and their nutrition could be classed as mixotrophic.

We have studied the action of *Scenedesmus sp.* J9A21, grown mixotrophically, on 4-pregnene-17α,21-diol-3,20-dione (Reichstein's Compound S). To an actively growing culture in an NZ amine(Type A)-starch medium was added Compound S at a level of 100 mg./l. of medium. After forty-eight hour fermentation with illumination, aeration and agitation, the entire batch was extracted with chloroform.

The concentrated chloroform extract was chromatographed over Florisil and eluted with methylene chloride containing progressively increasing amounts of methanol. From the 100% methylene chloride fractions there was isolated a non-ultraviolet-absorbing, non-steroidal, crystalline mixture which contained nitrogen. Later fractions, eluted with 1%, 2% and 3% methanol contained mixtures of ultraviolet-absorbing products with migration rates like those of authentic Compound S, hydrocortisone, 4-pregnene-11α,17α,21-triol-3,20-dione, 4-pregnene-6β,17α,21-triol-3,20-dione and 4-pregnene-15β,17α,21-triol-3,20-dione in the Shull system.[1] None of the steroid-containing fractions were crystalline.

The only steroidal product whose presence was established with certainty was 4-pregnene-6β,17α,21-triol-3,20-dione. This was accomplished by degrading with sodium bismuthate[2] a sample of the combined steroidal fractions from a preliminary Florisil chromatographic purification. The resulting mixture was then separated on a toluene-propylene glycol-Chromosorb W partition column. A single crystalline product was isolated, whose melting point, rotation, ultraviolet spectrum and infrared spectrum were in good agreement with that of 4-androstene-6β-ol-3,17-dione. This was presumed to

(1) G. M. Shull, abstracts of papers, 126th Meeting of the American Chemical Society, Sept. 12–17, 1954, New York, p. 9A.

(2) C. J. W. Brooks and J. K. Norymberski, *Biochem. J.*, **55**, 371 (1953).

arise from the product with mobility like 4-pregnene-6β,17α,21-triol-3,20-dione.

When *Scenedesmus sp.* was grown and incubated as before, but without addition of steroid, the metabolic mixture containing nitrogen, noted previously, was again isolated. This mixture was shown by paper chromatography in toluene-propylene glycol to contain two compounds, which could be stained with difficulty with ninhydrin, or more readily by the chlorine-iodide-starch method of Rydon and Smith.[3]

The mixture was resolved by successive chromatography over Florisil and partition in toluene-propylene glycol-Chromosorb W. There were isolated L - proline - L - leucinediketopiperazine (I) and L-proline-L-valinediketopiperazine (II), which were identified by degradation to the component amino acids by the Stein-Moore method.[4] Both I and II have been described previously; the former has been isolated from beef adrenals,[5] hog adrenals,[6] silk worm pupae[7] and fermentation broths from *Aspergillus* and *Penicillia sp.*[8] and has been prepared synthetically.[7,9] The latter has been isolated from silk worm pupae[7] and has been prepared synthetically.[7] The diketopiperazines were not formed by aeration of the medium used to grow *Scenedesmus sp.*, in the absence of the organism, and accordingly are presumed to be metabolic products.

EXPERIMENTAL[10]

Preparation of Scenedesmus sp. J9A21 culture. The algal culture, *Scenedesmus sp.* J9A21, is maintained on agar slants containing 0.1% NZ amine-type A and 0.1% soluble starch or in sterile soil-water medium, in the presence of calcium carbonate.

Three hundred-milliliter germination flasks containing 100 ml. of medium composed of 1% NZ amine-type A, and 1% glucose adjusted to *p*H 7.5 with sodium hydroxide are seeded from agar slants or soil-water tubes. The cultures are incubated on a New Brunswick type shaker rotating at 280 r.p.m. with a 1 1/2" stroke at 26°. Light is provided continuously during the growth stage. Incubation is continued until the packed cell volume is equal to 3 ml. per 100 ml. of medium.

(3) H. N. Rydon and P. W. G. Smith, *Nature*, 169, 922 (1952).

(4) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30, 1185 (1958).

(5) O. Wintersteiner and J. J. Pfiffner, *J. Biol. Chem.*, 111, 599 (1935).

(6) M. H. Kuizenga, J. W. Nelson, S. C. Lyster and D. Ingle, *J. Biol. Chem.*, 160, 15 (1945).

(7) A. Butenandt, P. Karlson and W. Zillig, *Z. Physiol. Chem.*, 288, 279 (1951).

(8) J. L. Johnson, W. G. Jackson and F. E. Eble, *J. Am. Chem. Soc.*, 73, 2947 (1951).

(9) E. Fischer and G. Reif, *Annalen*, 367, 126 (1908), A. Stoll, A. Hofman and T. Petrzilka, *Helv. Chim. Acta*, 34, 1544 (1951).

(10) All melting points are corrected. Analyses and optical data were obtained by the Physical Chemistry Dept. of the Schering Corp. We are indebted to Mr. Richard Wayne for the interpretation of the infrared spectra.

To this culture one can either add steroid directly for transformation, or the culture may be used as an inoculum at a 3% level for a fermentation tank as follows:

A glass fermentor having a working volume of 10 l. is charged with NZ amine-type A (100 g.), soluble starch (Difco) (500 g.), tap water (10 l.), and Larex (defoamer) (5 ml.), and sterilized for 30 min. at 121°. After cooling, the fermentor is inoculated and two 75-watt bulbs are mounted on two sides of the fermentor and 4 inches away from the tank. Air is sparged in at 1/2 volume per volume of medium per min. Agitation rate 300 r.p.m., temperature 26°.

After 48 hr. of growth or when the packed cell volume reaches 2%, 1 g. of Compound S in 5 ml. of dimethylformamide is added. The fermentation is continued for 48 hr. and then the whole broth is extracted with chloroform.

In runs made without steroid for the purpose of preparing the diketopiperazines I and II the aforedescribed cycle was carried out without the addition of steroid.

Transformation products of Reichstein's Compound S. The chloroform extract was concentrated to a small volume and chromatographed over 40 g. of Florisil. The defoamer was separated by washing with hexane and the steroidal mixture was eluted from the column with methylene chloride containing from 0% to 5% methanol. No satisfactory resolution was achieved. At least eight components absorbing ultraviolet light were noted on paper chromatograms of the various fractions. Components with the same mobilities as starting material, 4-pregnene-6β,17α,21-triol-3,20-dione, 4-pregnene-11β,17α,21-triol-3,20-dione, and 4-pregnene-11α,-17α,21-triol-3,20-dione were among those observed. In addition, in the earliest methylene chloride fractions there were observed small amounts of crystalline materials which did not absorb in the ultraviolet, and were apparently not derived from Compound S in that there was no stain with triphenyltetrazolium chloride and the infrared spectra were notably of a nonsteroidal type.

The fractions containing material absorbing in the ultraviolet were pooled and degraded with 20 g. of sodium bismuthate in 60 ml. of acetic acid and 30 ml. of water by agitation for 72 hr. at room temperature. Following filtration to remove sodium bismuthate and its reduction products, the filtrate was diluted with water and extracted with methylene chloride. The extracts were washed free of acetic acid, dried and concentrated to a residue (0.710 g.).

The residue was partitioned[11] on Chromosorb W (58 g.) bearing 58 g. of propylene glycol saturated with toluene. The steroidal mixture was dissolved in 5 ml. of the stationary phase which was then absorbed into a small amount of Chromosorb W. The resulting solid was placed at the top of the column with a layer of sand between it and the remainder of the column, and another layer above it. Elution with toluene saturated with propylene glycol afforded a series of 20-ml. fractions. Fractions 16 through 26 were homogeneous and crystalline, following evaporation of the solvent. The resulting product had the same migration rate as 4-androstene-6β-ol-3,17-dione in toluene-propylene glycol. After recrystallization from acetone-hexane there was isolated 10.7 mg., m.p. 185–190°, $[\alpha]_D^{25}$ +110° (chloroform), $\lambda_{max}^{CH_3OH}$ 235 mμ (ε 12,300), λ_{max}^{Nujol} 2.91 μ (OH), 5.76 μ (17-ketone), 5.98 μ (3 ketone) 6.20 μ (Δ^4). The infrared spectrum matched that of authentic 4-androstene-6β-ol-3,17-dione.[12]

No other crystalline fractions were observed from this partition chromatogram and all succeeding fractions were paper chromatographically heterogeneous.

Nonsteroidal metabolites of Scenedesmus sp. The chloroform extract from 40 l. of fermentation broth, prepared in the manner previously described, to which no steroid had

(11) *Cf.* H. L. Herzog, et al., *J. Org. Chem.*, 25, 2177 (1960).

(12) C. P. Balant and M. Ehrenstein, *J. Org. Chem.*, 17, 1587 (1952).

been added, was concentrated to a small volume. Paper chromatography of the concentrate in toluene–propylene glycol showed two components when the papers were developed according to Rydon and Smith. Chromatography over 300 g. of Florisil and elution with ether afforded two crystalline fractions, 8 and 9, which were homogeneous and identical with the less polar spot noted in the paper chromatogram of the mixture. Recrystallization from acetone-ether–hexane gave 0.271 g. of I (L-proline-L-leucinediketopiperazine), m.p. 160–162°; $[\alpha]_D^{25}$ −120° (dioxane), λ_{max}^{Nujol} 3.06 μ (N—H), 5.95 μ and 6.08 μ (amide carbonyl).

Anal. Calcd. for $C_{11}H_{18}O_2N_2$: C, 62.83; H, 8.62; N, 13.33; mol. wt. 210. Found: C, 63.11; H, 8.83; N, 13.84; mol. wt. (Rast) 221.

Fractions 10–16 (0.983 g.) eluted with ether were mixtures of I and II. Fractions 17–20 (0.557 g.) also eluted with ether, were predominantly II. The latter fractions were pooled and partitioned on 300 g. of Chromosorb W which held 300 g. of propylene glycol saturated with toluene. The pool of fractions 17–20 was placed on the partition column in the manner noted previously for steroid mixtures and eluted with toluene saturated with propylene glycol. Fractions 30–35 contained only the more polar component and these were pooled and crystallized from acetone-ether–hexane affording 0.075 g. of II (L-proline-L-valinediketopiperazine), m.p. 189–190°, $[\alpha]_D^{25}$ −164.5° (dioxane), λ_{max}^{Nujol} 3.06 μ (N—H), 5.96 μ and 6.12 μ (amide carbonyl).

Anal. Calcd. for $C_{10}H_{16}O_2N_2$: C, 61.45; H, 8.21; N, 14.3; mol. wt. 196. Found: C, 61.70; H, 8.33; N, 14.29; mol. wt. (Rast) 225.

Hydrochloric acid hydrolyses of I *and* II. A sample of 0.5 mg. of I was hydrolyzed with 1.0 ml. of 6*N* hydrochloric acid at 110° in a sealed tube for 24 hr. The hydrolysis mixture was concentrated to a residue which was dissolved in citrate buffer and analyzed according to Moore, Spackman and Stein[4] in a Phoenix Precision Instrument Co. (Phila., Pa.), Model K-5000 amino acid analyzer. Proline (0.27 mg.) and leucine (0.28 mg.) were identified by their elution volumes which were compared with authentic controls. Identities were confirmed by conversion to the dinitrophenyl derivatives which were prepared and characterized by paper chromatography according to Biserte.[13]

By the same procedure II (0.65 mg.) afforded proline (0.32 mg.) and valine (0.34 mg.) identified in the same way.

Optical forms of the amino acids. Samples of I and II were hydrolyzed according to the method of the preceding experiment and freed of hydrochloric acid by repeated concentration of the aqueous solutions. The residues were submitted to the action of D-amino acid oxidase (Worthington Biochemical) in the Warburg apparatus at 37°.[14] The results are recorded in Table I.

TABLE I

Substrate	Amount	Oxygen Consumed
DL-Leucine	15.3 μM	3.96 μM
DL-Valine	17.1 μM	4.67 μM
DL-Proline	17.4 μM	3.16 μM
I (hydrolyzed)	8.9 μM	0.27 μM
II (hydrolyzed)	9.7 μM	0.10 μM
Blank		±0.30 μM

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(13) G. Biserte and R. Osteux, *Bull. Soc. Chim. Biol.*, **33**, 50 (1951).

(14) S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, II, Academic Press, New York, 1955, p. 199.

Phenoxazines III. Dialkylaminoalkylphenoxazine-10 Carboxylates

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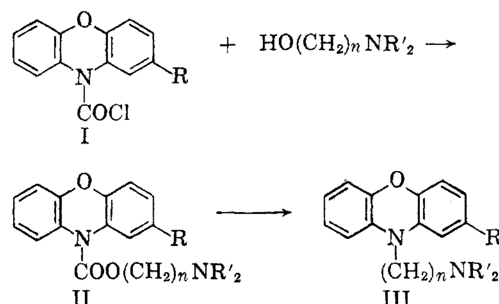
Various dialkylaminoalkyl esters of phenoxazine-10-carboxylic acid (II) were prepared by the reaction of the appropriate amino alcohol with phenoxazine-10-carbonyl chloride (I). This acid chloride was obtained by treating a toluene solution of phenoxazine with phosgene, a method which has already been used in the phenothiazine and carbazole series.[1]

These esters were decarboxylated by heating and the corresponding dialkylaminoalkylphenoxazines (III) were obtained.[2] An ester of 2-ethylphenoxazine-10-carboxylic acid (II. R = C_2H_5) was also treated in the same way. The aminoalkylphenoxazines (III) were identical with those prepared by other methods.[3]



EXPERIMENTAL[4]

Phenoxazine-10-carbonyl chloride (I. R = H). A 30% (w/w) solution (40 g.) of phosgene in toluene was added to a suspension of 11 g. of phenoxazine in 25 ml. of dry toluene in a Iena autoclave. The mixture was heated in an oil bath at 115° for 3 hr., under agitation with a magnetic stirrer. After cooling, the solution was evaporated to dryness under reduced pressure and the residue was crystallized in ethyl acetate. There was obtained 13.9 g. (94%), m.p. 139–141°.

3'-Dimethylaminopropylphenoxazine-10-carboxylate hydrochloride (II. R = H; NR'$_2$ = $N(CH_3)_2$; n = 3). A mixture of 7.5 g. (0.03 mole) of phenoxazine-10-carbonyl chloride and 3.15 g. (0.03 mole) of 3-dimethylaminopropanol in 30 ml. of dry benzene was heated on the steam bath for 17 hr.

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(2) This method was described for various phenothiazine- and carbazolecarboxylates: J. Schmitt, J. Boitard, P. Comoy, A. Hallot, and M. Suquet, *Bull. Soc. Chim. France*, 938 (1957); B.A.S.F. (H. Friederich, D. A. Grosskinsky, and A. Amann), German Pat. **939,630** (1956); *Chem. Abstr.*, **53**, 8172 (1959); Bayer, Brit. Pat. **808,049** (1959); *Chem. Abstr.*, **53**, 12312 (1959).

(3) H. Vanderhaeghe, and L. Verlooy, *J. Org. Chem.*, **26**, 3827 (1961).

(4) All melting points are uncorrected. The microanalyses were performed by Dr. A. Bernhardt, Mülheim (Ruhr) Germany.