Experimental

The nitroso compounds were prepared and purified by methods described in the literature.[5,13,14] The polarographic current–voltage curves were obtained with a Sargent Model XXI polarograph, using a conventional H-cell fitted with a standard calomel electrode. Buffer solutions were made up at 30° by mixing 50.00 ml. of 0.2000 *M* aqueous KH_2PO_4 and 29.63 ml. of 0.2000 *M* aqueous NaOH and diluting to the mark in a 100.0-ml. volumetric flask. The apparent pH of the resulting solutions was 7.00 ± 0.03. To 50.00 ml. of this buffer in a 100.0-ml. volumetric flask was added an aliquot of acetone stock solution of nitroso compound, and the mixture was diluted to the mark with acetone. Solutions were flushed in the H-cell with purified nitrogen for 3–6 min. before recording the polarogram. Temperatures were controlled to 30 ± 0.1° by immersing the H-cell in a constant-temperature bath. Various capillary drop times and concentrations between 1×10^{-3} and 5×10^{-5} *M* gave the same half-wave potentials. The dropping mercury electrode at a pressure of 60.0 cm. had a drop time of 3.60 sec. (open circuit) in 50% acetone–50% pH 7 phosphate buffer at 30°. The value of m was 2.69 mg. sec.$^{-1}$ with a calculated value of $m^{2/3}t^{1/6}$ of 2.40 mg.$^{2/3}$ sec.$^{-1/2}$.

The spectrophotometric measurements were carried out with a Beckman DU equipped with a double set of thermospacers through which water from a constant-temperature bath was circulated. A hole through the lid of the cell compartment allowed a thermometer to be placed so that its bulb was immediately adjacent to the cell containing the solution. Matched 1.00-cm. silica cells were used and temperatures were maintained constant to within ±0.05°. Optical densities were determined at or near the wave length of maximum absorption due to the characteristic[4] n–π transition of aromatic nitroso monomers. Optical densities were reproducible to within ±0.5% or better except at the lowest concentrations where reproducibility was somewhat less satisfactory. ϵ_0, the molar extinction coefficient, was determined by linear extrapolation (Ostwald dilution law) of the usual[4] ϵ *vs.* $\epsilon^2 \cdot C^0$ plots (where C^0 is concentration of total nitroso compound, all figured as monomer) to zero concentration. The resulting values of ϵ_0 were then used in the calculation of equilibrium constants and per cent monomer at 20°. The optical densities (1-cm. cell, 775 mμ) and concentrations in benzene (moles/liter) for 2,4,6-trichloronitrosobenzene were 0.6720, 0.0250; 0.3980, 0.0125; 0.2250, 0.00625; 0.1223, 0.003125 (at 25°); 0.5150, 0.0200 (at 20°), which led to ϵ_0 = 43.7 l./mole-cm. and K_c = 3.38×10^{-2} moles/l. (at 20°). For 2,6-dichloronitrosobenzene (1-cm. cell, 790 mμ) in benzene the corresponding quantities were 0.4530, 0.0200; 0.2185, 0.0100; 0.1645, 0.0050; 0.0925, 0.0025 (at 30°); 0.2590, 0.0125 (at 20°), giving ϵ_0 = 44.0 l./mole-cm. and K_c = 1.048×10^{-2} moles/l. (at 20°).

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A New Preparation of Gentiobiose[1]

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Determination of the structure of complex carbohydrates by chemical and biochemical methods has necessitated the preparation of oligosaccharides for reference purposes and for use as substrates to ascertain the action pattern of carbohydrases.[2] Oligosaccharides have been obtained by direct extraction of natural products, by acid hydrolysis of polysaccharides, by chemical or enzymic synthesis, and also by enzymic degradation of polysaccharides.

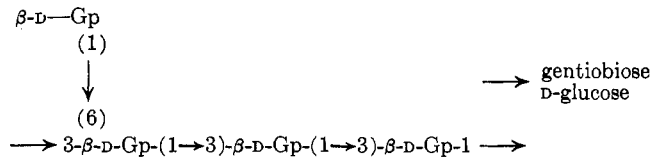
Although gentiobiose can be isolated directly from the gentian root[3,4] and prepared synthetically by chemical[5-7] and enzymatic methods,[8,9] it remains nevertheless relatively inaccessible and expensive.

The recent observation[10] that a β-D-(1→3)-glucanase, isolated from culture filtrates of *Basidiomycete* sp. QM 806,[11] completely hydrolyzes the extracellular D-glucan elaborated by a fungus of the Fungi imperfecti group to give 1 molecular proportion of gentiobiose and 2 molecular proportions of D-glucose suggested that this might form the basis of a new method for making gentiobiose.

It is of interest to note that the β-D-glucan produced by *Claviceps purpurea* behaves similarly when treated with an *exo*-laminarase preparation to give gentiobiose and glucose in a molar ratio of about 1:3.[12] Likewise sclerotan, a glucan generated by *Sclerotina libertiana*, gives glucose and gentiobiose when treated with an enzyme, a β-D-(1→3)-glucanase, isolated from cultures of this organism.[13,14]

The imperfecti D-glucan referred to above has been shown[10] to be composed of a chain of β-(1→3)-linked D-glucose units, one out of every three of which is attached to a single glucose unit by a (1→6) linkage (Scheme I). This polysaccharide was allowed to react

SCHEME I



with the *Basidiomycete* β-D-(1→3)-glucanase until maximum reducing power had been attained. The mixture of gentiobiose and glucose was then separated by charcoal column chromatography[15] either before or after treatment with yeast and the gentiobiose was crystallized as the methanolate. Since the glucan used in this work is only one of a number of structurally related polysaccharides it seems that any one of them should provide an excellent source of gentiobiose.

Experimental

Preparation of β-D-(1→3)-glucanase.—*Basidiomycete* sp. QM 806 was grown in a modified medium similar to that described by Reese and Mandels[8] with the exception that glucose rather than starch was used as the source of carbohydrate. The crude culture filtrate (1 l.) was centrifuged, concentrated 50-fold *in vacuo*

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at 40° (bath temperature), filtered through a Millipore filter, dialyzed, and reconcentrated to 20 ml. The solution was found to contain 240 units of activity/ml. when assayed in the manner described by Nelson, *et al.*[2]

Hydrolysis of the (1→3,1→6)-Linked D-Glucan.—A portion of the enzyme solution (5 ml.) was added to the polysaccharide (5 g.) dispersed in 0.05 *M* acetate buffer (1.0 l.), pH 4.8. The hydrolysis was allowed to proceed at 50°, samples being removed after 1, 2, 4, 6, 10, and 21 hr. The samples were heated in a steam bath for 2 min. to deactivate the enzyme and centrifuged; the reducing sugar content was determined by the method of Nelson–Somogyi[16] and a constant value was obtained after 4 hr.

The hydrolysis was terminated after 21 hr. by autoclaving for 2 min. at 110–120°. A small amount of inorganic material (160 mg.) was removed by centrifugation after which the solution was neutralized by the addition of 10% sodium hydroxide and concentrated *in vacuo* to approximately 50 ml.

Alternately, the hydrolysate was treated with washed baker's yeast (5 g.). The disappearance of glucose by this somewhat more time-consuming procedure was followed by thin layer chromatography on Kieselguhr G according to Stahl.[17] The cells were removed by centrifugation, and the solution was neutralized and concentrated to approximately 50 ml.

Isolation of Gentiobiose by Charcoal Column Chromatography.—The hydrolysate (50 ml.) before or after treatment with yeast was added to a column of charcoal[15] (2.5 × 18 cm.) and washed successively with water (7.0 l.), 5% ethanol (4.0 l.), and finally with 10% ethanol (2.0 l.), the eluate being collected in 500-ml. fractions. The fractions were concentrated *in vacuo* and the contents were analyzed by thin layer and paper chromatography. The 10% aqueous ethanol eluate contained the gentiobiose which was obtained as a chromatographically pure sirup (1.68 g.) by evaporation of the solvent *in vacuo*. The sirup was dissolved in refluxing absolute methanol and the product (1.41 g.) crystallized by cooling: m.p. 84° dec.,[9] $[\alpha]^{23.5}D$ +13.0° (*c* 33, water) (after 15 min.), changing in 24 hr. to +8.4° (equilibrium value); lit.[12,18,19] m.p. 85–86°, $[\alpha]^{20}D$ +21.4 → +8.7° in water.

Acetylation of a small portion of the product with acetic anhydride and anhydrous sodium acetate[4] yielded β-gentiobiose octaacetate, m.p. and m.m.p. 191° (after recrystallization from methanol), lit.[12,18] m.p. 193°.

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Phenylcyclobutane Amino Acids

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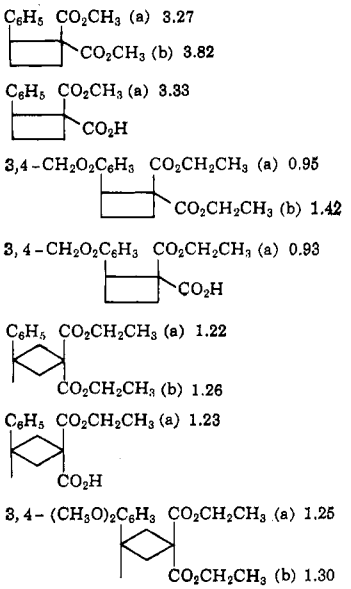
Conversions of 2- and 3-phenylcyclobutane-1,1-dicarboxylic acids[1a,b] and of alkoxy-substituted derivatives to the corresponding 1-amino-2- and -3-arylcyclobutane-1-carboxylic acids have been carried out by partial hydrolysis of the dicarboxylate esters of these acids, and Curtius degradation of the carboxyl group of the resulting monoalkyl esters, followed by hydrolysis of the remaining ester group.

In the 2-aryl series, sterically homogeneous monoalkyl esters were obtained in good yields. The assumption that the more readily hydrolyzed ester group of the dicarboxylate esters would be *trans* to the sterically hindering aromatic moiety was supported by the greater interference of the bulkier 2-(3,4-methylenedioxyphenyl) compared with the 2-phenyl group. Under conditions which hydrolyzed the 2-phenylcyclobutane-1,1-dicarboxylate ester completely, the 2-(3,4-methylenedioxyphenyl) ester was only hydrolyzed partially. In this methylenedioxy series, the Curtius degradation was also interrupted at intermediate stages; alkaline hydrolysis of 1-carbethoxy-2-(3,4-methylenedioxyphenyl)cyclobutane 1-isocyanate led only to an ester urea, and refluxing the corresponding ester carbamate with hydrochloric acid gave the amino ester instead of the amino acid.

(1) (a) C. Beard and A. Burger, *J. Org. Chem.*, **26**, 2335 (1961); (b) **27**, 1647 (1962).

TABLE I

N.M.R. CHEMICAL SHIFTS FOR METHYL PROTONS OF SOLUTIONS OF ALKYL ARYLCYCLOBUTANE-1,1-DICARBOXYLATES[a]



[a] Relative to tetramethylsilane; given in parts per million.

The amino group of the amino acids prepared by Curtius degradation must have the same configuration (*trans*) as the carboxyl group of these half esters.

In the 3-aryl-substituted series, less steric hindrance may be expected and, therefore, less difference in the rate of the hydrolysis of the two ester groups. Indeed, oily mixtures of half esters were obtained upon hydrolysis of the dicarboxylate esters, and only in one case could a pure stereoisomer be elaborated in smaller yields.

Models of the ethyl hydrogen 2-phenylcyclobutanedicarboxylate esters show that in the favored conformation the ethyl group is perpendicular to the benzene ring. If the ester group is in the plane of the benzene ring, its carbonyl oxygen interferes with the orthohydrogens. In the n.m.r. spectrum, the *cis* ethyl protons should appear more shielded and thus upfield with respect to *trans* ethyl protons. This has actually been observed (Table I).

In the case of the ethyl hydrogen 3-phenylcyclobutane-1,1-dicarboxylates, the chemical shifts of the *cis* and *trans* protons of the methyl groups are very close as expected from the increased distance of the aromatic ring to either of these groups (Table I). The only pure isomer obtained was probably the *trans* carboxy compound.