Anal. Calcd. for $C_{13}H_{13}O_5N$ (263.2): C, 59.3; H, 5.0; N, 5.3. Found: C, 59.4; H, 5.1; N, 5.3.

α-Phthalimido-β-ethoxypropionyl Chloride (IV).—III, 14.4 g., was heated under refluxing conditions for 1 hour with 30 ml. of thionyl chloride, the reaction mixture freed of excess thionyl chloride by distillation and repeated co-distillation with benzene, and the residue recrystallized from a mixture of 60–80° ligroin and benzene to give 10.0 g. of IV, m.p. 70–72°.

Anal. Calcd. for $C_{13}H_{12}O_4NCl$ (281.7): C, 55.4; H, 4.3; Cl, 12.6. Found: C, 55.5; H, 4.3; Cl, 12.6.

α-Phthalimido-β-ethoxyethyl Diazomethyl Ketone (V).—A solution of 13.75 g. of IV in 400 ml. of dry ether was slowly added, at 0–5°, to 500 ml. of an ethereal solution containing *ca.* 7 mole equivalents of diazomethane, the reaction mixture maintained at 0–5° for 2 hours and then allowed to reach room temperature before the excess diazomethane and solvent was removed by distillation. The residue was recrystallized from a mixture of ethyl acetate and 60–80° ligroin to give 9.7 g. of V, yellow prisms, m.p. 87.5–89°.

Anal. Calcd. for $C_{14}H_{13}O_4N_3$ (287.3): C, 58.5; H, 4.6; N, 14.6. Found: C, 58.6; H, 4.7; N, 14.5.

Methyl β-Phthalimido-γ-ethoxy-*n*-butyrate (VI).—To a solution of 10 g. of V in 100 ml. of methanol was added a small amount of freshly prepared silver oxide suspended in methanol and the reaction mixture heated under refluxing conditions. When the evolution of nitrogen had subsided a further quantity of the silver oxide suspension was added and the process repeated until no further reaction was observed. The precipitate present in the reaction mixture was removed by centrifugation, washed with methanol and the combined supernatant and washings freed of solvent. The residual oil was dissolved in chloroform, the solution filtered, the filtrate evaporated to dryness and the residue distilled to give 8.3 g. of a yellow viscous oil, b.p. *ca.* 170° (0.1 mm.), which crystallized upon standing to give VI, m.p. 66–67°, after two recrystallizations from methanol.

Anal. Calcd. for $C_{15}H_{17}O_5N$ (291.3): C, 61.8; H, 5.9; N, 4.8. Found: C, 62.3; H, 5.9; N, 4.6.

β-Phthalimido-γ-ethoxy-*n*-butyric Acid (VII).—A mixture of 3.25 g. of VI, 7 ml. of concentrated hydrochloric acid and 150 ml. of water was heated under refluxing conditions for 3 hours, the hydrolysate cooled, extracted with benzene, the extract freed of solvent and the residue recrystallized first from aqueous ethanol and then from a mixture of benzene and 60–80° ligroin to give 2.3 g. of VII, m.p. 104–106°.

Anal. Calcd. for $C_{14}H_{15}O_5N$ (277.3): N, 5.0. Found: N, 4.7.

α-Bromo-β-phthalimido-γ-butyrolactone (VIII).—To an intimate mixture of 2.0 g. of VII and 0.1 g. of red phosphorus was added dropwise and with cooling 1.2 ml. of bromine. After all of the bromine was added the reaction mixture was heated on a steam-bath for 6 hours. Water was then added, the excess bromine removed by heating, and the colorless aqueous solution cooled to give a crystalline precipitate of VIII which was suspended in 15 ml. of boiling ethanol. The hot ethanol solution was decanted from the undissolved portion of the precipitate and cooled to give 0.4 g. of product m.p. 143–167°. This product was alternately recrystallized from methanol and from ethanol to give the more soluble and lower melting diastereoisomeric mixture (VIIIa) of VIII, m.p. 185°.

Anal. Calcd. for $C_{12}H_{18}O_4NBr$ (310.1): C, 46.5; H, 2.6; N, 4.5; Br, 25.8. Found: C, 46.5; H, 2.6; N, 4.5; Br, 25.9.

The crystalline residue remaining after the initial extraction with a limited amount of ethanol was dissolved in a relatively large volume of boiling ethanol, and the solution cooled to give 0.5 g. of the less soluble and higher melting diastereoisomeric mixture (VIIIb) of VIII, m.p. 196–201° with decomposition after one additional recrystallization from ethanol.

Anal. Calcd. for $C_{12}H_8O_4NBr$ (310.1): C, 46.5; H, 2.6; N, 4.5; Br, 25.8. Found: C, 46.8; H, 2.5; N, 4.7; Br, 26.1.

DL-Isoserine.—A mixture of 4.0 g. of α-bromo-β-phthalimidopropionic acid (IX), 5.2 g. of barium carbonate and 18 ml. of water was heated under refluxing conditions for 1 hour. The reaction mixture was cooled, the excess barium carbonate collected and thoroughly washed with hot water, the combined filtrate and washings freed of barium ion with sulfuric acid, the aqueous solution evaporated to a small volume, cooled, the precipitated phthalic acid collected, the filtrate made alkaline with ammonium hydroxide and evaporated to dryness *in vacuo*. The residue was dissolved in water, the solution filtered, evaporated to a small volume, and cooled to give 0.55 g. of DL-isoserine, m.p. 237° with decomposition.

α,γ-Dihydroxy-β-amino-*n*-butyric Acid (X).—VIIIb, 0.8 g., was dissolved in a solution of 0.95 g. of barium oxide in 40 ml. of water, the solution saturated with carbon dioxide and then heated under refluxing conditions for *ca.* 1 hour. To the cooled reaction mixture was added 0.8 g. of concentrated sulfuric acid in 3 ml. of water, the precipitate collected and washed with hot water, and the combined filtrate and washings evaporated *in vacuo* to a small volume. The phthalic acid which separated upon cooling was collected, the filtrate freed of barium ion by the careful addition of dilute sulfuric acid, the filtrate again concentrated *in vacuo* and a second crop of phthalic acid collected. To the filtrate was added 1 g. of silver carbonate, the mixture heated to boiling, the precipitate removed by centrifugation, the supernatant liquid again concentrated *in vacuo*, the precipitate removed by centrifugation, the supernatant liquid saturated with hydrogen sulfide, the silver sulfide collected and the supernatant liquid evaporated *in vacuo* to give X, granular crystals, m.p. 214° with decomposition.

Anal. Calcd. for $C_4H_9O_4N$ (135.1): C, 35.6; H, 6.7; N, 10.4. Found: C, 35.7; H, 6.5; N, 10.1.

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Substrate and Cosubstrate Requirements for Enzymatic Transfructosidation[1]

BY JOHN H. PAZUR

RECEIVED JUNE 26, 1953

Transfructosidation action of enzymes from yeasts[2-4] and molds[5,6] on fructosyl oligosaccharides results in the synthesis of new carbohydrates. Sucrose and raffinose have been found to function as substrates and cosubstrates (acceptor molecules) for the transfructosidase of *Aspergillus oryzae* while fructose and inulobiosylglucose function as cosubstrates only. The enzyme is without action on turanose and melezitose.[5,6] In the studies being reported, three new fructosyl compounds (inulobiose,[7] stachyose[8] and planteose[9]) have been tested as potential substrates for the transfructosidase of *Aspergillus oryzae*. The possibility that these oligosaccharides may function as acceptor molecules in the transfer of fructose units of sucrose has also been investigated.

The transfructosidase was allowed to act on solutions of the fructosyl compounds at room temperature. Aliquots of the digestion mixtures were removed at various time intervals and heated at 100° for 5 minutes to arrest enzyme action. The quali-

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tative composition of these aliquots was determined by paper chromatographic procedures.

Inulobiose (1-β-fructofuranosyl-D-fructose) was disproportionated by transfructosidase to yield fructose and a new reducing fructosyl compound. Since the enzyme transfers fructose units from substrates to the 1-position of fructose moieties of cosubstrates,[5] it is probable that the synthesized compound is inulotriose, the trisaccharide of the inulin series.[7] Such a structure is also indicated by the identical R_f values obtained for inulotriose and for the synthesized compound.

Although stachyose did not function as a substrate for the enzyme, it did, nevertheless, partake in transfructosidation as a cosubstrate. An enzymatic digest of stachyose and sucrose contained the disproportionation products of sucrose as well as a new fructosyl compound. The new compound was non-reducing and moved on paper at a rate which would be expected for a pentasaccharide with a fructosylstachyose structure. Inclusion of C^{14}-sucrose in the digestion mixture resulted in the synthesis of radioactive fructosylstachyose. The radioactive compound was isolated, in pure form, and hydrolyzed to fructose and manninotriose. Activity measurements on the hydrolytic products showed that the fructose was the radioactive portion of the fructosylstachyose.

Planteose, the third oligosaccharide tested, did not function either as a substrate or cosubstrate for the transfructosidase. Apparently two substitutions on the fructose moiety of planteose eliminate it from the range of substrates or cosubstrates for the transferring enzyme of *Aspergillus oryzae*.

Experimental

Enzymatic Digests of Oligosaccharides.—A sample of 1 ml. of 0.04 *M* solution of the carbohydrate (inulobiose, planteose or stachyose) was mixed with 1 ml. of a 1% solution of enzyme concentrate.[10] Aliquots of 0.2 ml. were removed from the digest at time intervals of 0, 3, 6, 12 and 24 hours. The enzyme was destroyed by heat and the aliquots were examined for reaction products by multiple ascent paper chromatography.[5]

The compounds in the digest of inulobiose were resolved by two ascents of the solvent. The apparent R_f values[11] under these conditions were: 0.64 (fructose), 0.49 (inulobiose) and 0.36 (inulotriose). On prolonged enzyme action, the inulobiose was converted to fructose.

Some hydrolysis of the sucrosyl linkage in planteose and stachyose occurred after enzymolysis for 24 hours. There was, however, no evidence of transfructosidation in these digests. Under the same conditions new compounds were produced from sucrose and raffinose by the transferring enzyme.[5]

Enzymatic Digests of Mixtures of Oligosaccharides.—Digests of 0.5 ml. of 0.04 *M* sucrose solution, 0.5 ml. of 0.04 *M* solution of planteose, stachyose or inulobiose and 1 ml. of enzyme solution were prepared. Samples were obtained at time intervals of 0, 3, 6, 12 and 24 hours as above. Examination of these samples showed that disproportionation of sucrose had occurred, that planteose did not function as a cosubstrate, and that stachyose and inulobiose did function as cosubstrates. The apparent R_f values (6 ascents of solvent) of the reducing and non-reducing compounds in an enzymolysate of stachyose and sucrose were: 0.92 (fructose), 0.87 (glucose), 0.80 (sucrose), 0.61 (inulobiosylglucose), 0.48 (inulotriosylglucose), 0.22 (manninotriose), 0.15 (stachyose) and 0.07 (fructosylstachyose).[11] The R_f values (two ascents of solvent) of the reducing compounds in the digestion mixture of inulobiose and sucrose were identical with those listed in the preceding section.

Enzymatic Digest of C^{14}-Sucrose and Stachyose.—A sample of 2 mg. of C^{14}-sucrose (total activity *ca.* 6,000 c.p.m.) was dissolved in 0.1 ml. of a solution of sucrose (0.02 *M*) and stachyose (0.02 *M*). To this solution 0.1 ml. of the enzyme was added. The mixture was allowed to stand at room temperature for 18 hours. Two aliquots of 0.05 ml. of the digest were placed on a paper chromatogram. One-half of the developed chromatogram was sprayed with phloroglucinol reagent. On heating the sprayed chromatogram, the areas at which the fructosyl compounds are located appear as brown spots. These areas were cut from the chromatogram and their radioactivities determined in a conventional counting apparatus (Nuclear Scaler model no. 166). The activities of the compounds counted were as follows: fructose + glucose 792 c.p.m., sucrose 550 c.p.m., stachyose 2 c.p.m., and fructosylstachyose 144 c.p.m. The fructosylstachyose from the unsprayed portion of the chromatogram was extracted with water, taken to dryness, dissolved in 0.1 ml. of 0.05 *N* hydrochloric acid and heated at 80° for 30 minutes. The hydrolytic products (fructose and manninotriose) were separated on paper. The radioactivities of the products were found to be: fructose 132 c.p.m. and manninotriose 4 c.p.m.

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(10) The enzyme concentrate of *Aspergillus oryzae* was supplied by Takamine Laboratory, Inc., Clifton, N. J.

(11) Since multiple ascent chromatography was used, apparent R_f values are recorded. These values are obtained by dividing the height to which the individual compounds have moved by the total height of the paper strip. Differences in apparent R_f values of 0.05 are sufficient to give distinctly separated spots.

Reduction of Fluorine-containing Esters by Grignard Reagents

BY O. R. PIERCE, J. C. SIEGLE AND E. T. MCBEE

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It has been previously reported[1,2] that esters of perfluorinated acids could be reduced by Grignard reagents which contain β-hydrogen atoms to form the secondary alcohol corresponding to the alkyl group of the Grignard reagent. The explanation offered for this phenomenon is based on the ready reduction, demonstrated independently,[1,2] of the ketone formed as an intermediate in the reaction.

In this Laboratory, the reducing tendency of fluorine-containing esters has been employed as a means of preparation of secondary alcohols directly from the esters. By use of isopropylmagnesium halide as the reducing agent, it is possible to prepare secondary alcohols containing methyl, ethyl or phenyl groups from the corresponding Grignard reagents. The reactions are conducted employing a mixture of isopropyl and the alkyl or aryl Grignard desired. The experimental results are summarized in Table I.

It is interesting to note that as the length of fluorinated carbon chain of the ester increases the yield of reduction products is increased which is in accordance with previous observations in the perfluorinated aldehyde series.[1] No significant difference was observed between the reaction of a methyl or ethyl ester. Perhaps a more significant factor is the difference in reactivity observed between the isopropylmagnesium halide and the other Grignard reagents used with the former considerably less reactive under these conditions. This difference was not apparent when the reactions

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