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Note

A colorimetric method for the estimation of 2-deoxy-3-*C*-methyl-branched sugars

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The extensive research on the chemistry of antibiotics has led to the discovery of numerous new sugars, many of which possess unusual structures[1,2]. Among these, mycarose (2,6-dideoxy-3-*C*-methyl-L-*ribo*-hexose)[3], olivomycose (2,6-dideoxy-3-*C*-methyl-L-*arabino*-hexose)[4], axenose (2,6-dideoxy-3-*C*-methyl-L-*xylo*-hexose)[5], and D-evermicose (2,6-dideoxy-3-*C*-methyl-D-*arabino*-hexose)[6] are unique, as their structures contain both two deoxy (at C-2 and C-6) and methyl-branched (at C-3) moieties. Despite dramatic advances made in recent years in understanding the pathways of natural-product biosynthesis, little is known about the biosynthetic formation of these unusual sugar molecules. During the course of our investigation designed to elucidate the biosynthesis of 2,6-dideoxy-3-*C*-methylhexoses at the molecular level, the need arose for a sensitive and specific test for this class of sugars, to be used as an enzyme-activity assay to facilitate the protein purification. A close examination of the structures of these compounds allowed us to envisage that the 2-thiobarbituric acid (TBA)–aldehyde reaction, which has been used for the estimation of 2- (ref. 7) as well as 3-deoxyhexoses[8], might equally well be applicable to the quantitative determination of 2-deoxy-3-*C*-methylhexoses, including the 6-deoxy derivatives thereof.

The TBA test is a method commonly used for measuring oxidative changes in foods containing unsaturated fatty acids[9]. The red pigment produced in the reaction between TBA and oxidized lipids has been suggested to be a condensation product of two molecules of TBA with one molecule of malonaldehyde[10]. The malonaldehyde is believed to be derived from some decomposition product of the oxidized unsaturated fatty acids. However, this common belief has never been unequivocally substantiated[9,11]. Because malonaldehyde is also generated in the periodate oxidation of 2- and 3-deoxyhexoses, spectrophotometric analysis of the resulting

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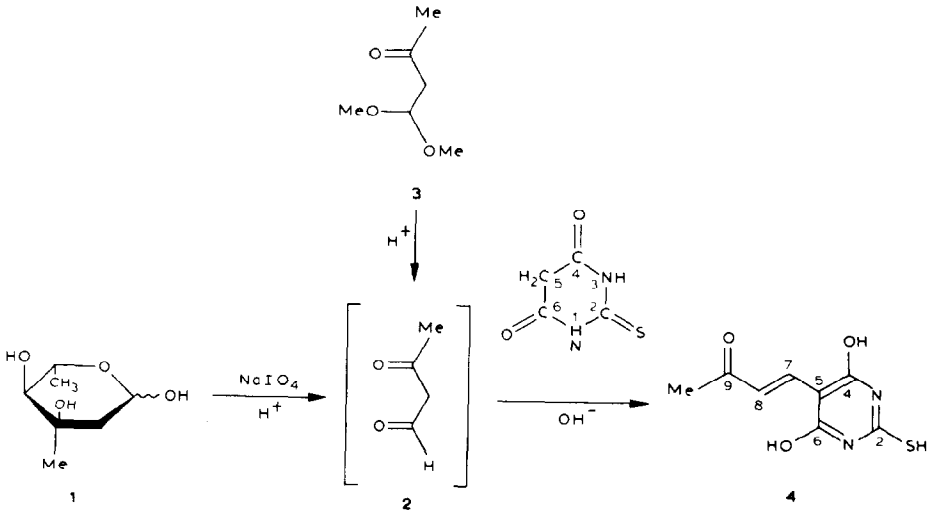
red chromogen, which is most likely to be the malonaldehyde–TBA complex, has been shown to provide a quantitative measure of these deoxy sugars. The proposed method to detect the existence of 2,6-dideoxy-3-*C*-methylhexose (*e.g.*, **1**) follows the same principle, and is based on periodate oxidation and estimation of the ensuing acetylacetaldehyde (**2**) with TBA (see Scheme 1). Described herein are the results of this assay performed on a sample of L-mycarose (**1**). The unambiguous characterization of the chemical nature of the resulting chromogen is also reported.



The assay was initiated by the incubation of L-mycarose (**1**) with periodate reagent. Upon treatment with 2-thiobarbituric acid in acidic solution, the resulting oxidatively degraded products gave rise to a characteristic chromophore having an absorption maximum at 372 nm (see Fig. 1). Further analysis showed that a linear relationship existed between the concentration of L-mycarose and the optical absorbance at 372 nm (see Fig. 2). However, the intensity of the color development varied with the concentration of the TBA reagent and the incubation temperature. It was later established that incubation with diluted TBA reagent at room temperature seemed to give less complication, and the resulting chromogen was obtained in optimal yield. Isolation and characterization of this pigment from the assay mixture yielded a compound identical with that obtained on allowing acetylacetaldehyde (**2**) to react with an excess of TBA. The adduct of TBA and acetylacetaldehyde was prepared, and purified according to the procedures described by Sinnhuber *et al.*[12], except that acetylacetaldehyde 1,1-dimethyl acetal (**3**) was used in the preparation.

However, co-produced with the major product **4** in the synthesis was an unidentified, minor chromogen, believed to be the 1:2 aldehyde–TBA adduct. This minor adduct was expected to possess a highly chromophoric group, and was found to give a red color having a distinct visible spectrum with an absorption maximum

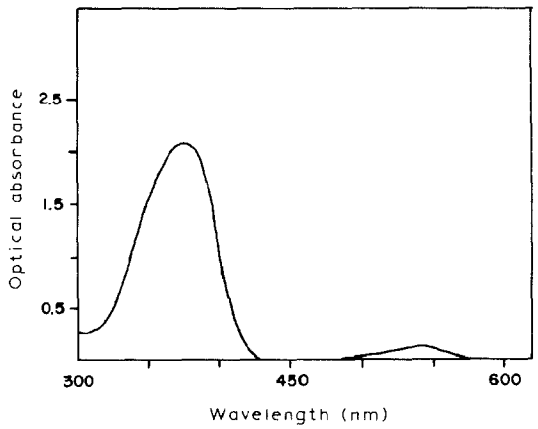


Fig. 1. Chromophore absorption spectrum for the acetylacetaldehyde–TBA adduct.

at 542 nm (see Fig. 1). Due to the co-existence of this contaminating, albeit dominant, chromophore, the color of the product mixture was mahogany red. Because the mixture of products was almost insoluble in most common solvents, and the contaminating adduct was present in only minute proportion, the major product **4** was subjected to chemical analysis without purification. The dark-red crystals that formed decomposed above 300°, and gave a prominent molecular-ion peak ($M^+ + H$) at 213.0349 (f.a.b.-m.s.) which correctly analyzed for $C_8H_8N_2O_3S$ (M^+). Its u.v.–visible spectrum in aqueous solution exhibits an absorption maximum at 372 nm (ε_{mM} 38.64), which is identical to that of the TBA adduct derived from degradation of L-mycarose. The F.t.-i.r. spectrum (KBr) clearly revealed bands characteristic of OH and NH stretching (3445 cm^{-1}), SH stretching (2557 cm^{-1}), enone carbonyl stretching (1665 cm^{-1}), amide carbonyl stretching

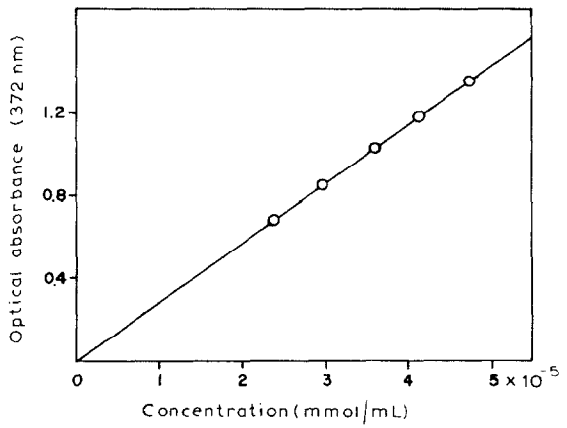


Fig. 2. Relationship between concentration of acetylacetaldehyde and optical absorbance at 372 nm.

(1612 cm^{-1}), and C–N vibration of N–C–S (1515 cm^{-1}). These data suggested that the pigment is highly conjugated and may be in a quinonoid form.

Examination of the ^{1}H-n.m.r. and ^{13}C-n.m.r. spectra of this adduct in Me_2SO-d_6 gave considerable insight into its chemical nature. Because only seven signals are discernible in the ^{13}C-n.m.r. spectrum, the adduct must possess a certain degree of symmetry within its structure. Among the four quarternary-carbon signals between 190 and 100 p.p.m., two of them, at 189.2 and 176.6 p.p.m., can based on their chemical shifts be assigned to the carbonyl carbon atoms of the enone and the amide, respectively. The broadened peak at 162.1 p.p.m. can be ascribed to the N=C–S of a thioamide group. The remaining quarternary signal, at 100.4 p.p.m., may be attributed to the sp^2 ring-carbon atom (C-5) to which the side chain is attached. These results strongly suggested that the malonylthiourea ring is still intact. The existence of an (*E*)-enone was revealed by the characteristic AB quartet ($J_{7,8}$ 14.5 Hz) at 8.00 and 7.07 p.p.m. in the ^{1}H-n.m.r. spectrum, assignable to the alkenic protons on a conjugated *trans*-disubstituted double bond. The presence of an acetyl group is clearly indicated by the sharp three-proton singlet at 2.18 p.p.m. These assignments were further supported by the ^{13}C-n.m.r. resonances of two methine carbon atoms, at 148.6 and 109.4 p.p.m., and a methyl carbon signal at 22.5 p.p.m. Thus, the structure of this chromogen was established as being **4**.

The preceding evidence clearly revealed that the adduct formed in the periodate oxidation–TBA coupling assay is compound **4**, which can be quantified spectrophotometrically. Formation of the 1:1 adduct of TBA and acetylacetaldehyde may be initiated by nucleophilic attack involving C-5 of TBA on to the aldehydic carbon atom of the ensuing acetylacetaldehyde, followed by dehydration. Unlike the coupling between TBA and a dialdehyde, which always produces a 2:1 adduct, the reaction of TBA with acetylacetaldehyde leads to the formation of a 1:1 adduct only. Because the extent of conjugation of the resulting chromophore is shortened in the 1:1 adduct, compound **4** exhibits an absorption maximum at an unusually short wavelength. Although TBA is a very versatile reagent and will react with many carbonyl compounds, the λ_{max} value at 372 nm for the acetylacetaldehyde–TBA adduct is well separated from the absorption maximum, at 532 nm, of the malonaldehyde–TBA adduct derived from autoxidation of polyunsaturated fatty acids[13], or periodate degradation of 2-, 3-, or 4-deoxyhexoses[7,8]. The unique u.v. absorption of **4** is also distinct from the λ_{max} value of 549 nm of the β-formylpyruvic acid–TBA adduct produced through periodate oxidation of sialic acid or *N*-acetylneuraminic acid[14,15]. Thus, no complication is expected in applying this assay to a natural incubation mixture. Because formation of acetylacetaldehyde during periodate oxidation is characteristic for the parent structural skeleton of 2,6-dideoxy-3-*C*-methyl-branched sugars, and for 2-deoxy-3-*C*-alkyl-branched sugars in general, this colorimetric method provides a unique, reliable, and interpretable test by which to assay the formation or existence, or both, of this class of sugar molecules.

EXPERIMENTAL

Preparation of pigment. — The procedure developed by Sinnhuber *et al.*[12] for the preparation of the malonaldehyde–TBA complex was used for the present synthesis. TBA (3.2 mmol) and acetylacetaldehyde 1,1-dimethyl acetal (1.6 mmol) in 12% hydrochloric acid (62.5 mL) were boiled under reflux for 60 min. A bright vermilion-colored product formed at once. Owing to the contamination by an unidentified, minor adduct which gave an absorption at 542 nm, the pigment separated from the acid solution as dark-red crystals. These were filtered off on a sintered-glass filter-funnel with suction, successively washed with 0.6M hydrochloric acid (100 mL), hot water (briefly), ethyl alcohol (20 mL), 1:1 alcohol–ethyl ether (100 mL), and ethyl ether (100 mL). As TBA is slightly soluble in ethyl ether, some of it may be removed by continuous washing with this solvent. The dark-red crystals were dried *in vacuo* overnight at 60°, the yield was 86%. This product was only slightly soluble in alcohol, but readily soluble in Me_2SO, TFA, and caustic alkalis; $\lambda_{max}^{H_2O}$ 372 nm (ε_{mM} 38.64); a peak at 542 nm is attributable to the absorption of the minor adduct; ^{1}H-n.m.r. data: δ 8.00 (d, 1 H, $J_{7,8}$ 14.5 Hz), 7.07 (d, 1 H, $J_{7,8}$ 14.5 Hz), and 2.18 (s, 3 H, CH_3–C=O); ^{13}C-n.m.r. data: δ 189.2 (C-9), 176.6 (C-4–C-6), 162.1 (C-2), 148.6 (C-7), 109.4 (C-8), 100.4 (C-5), and 22.5 (C-10); high-resolution f.a.b.-m.s.: Calc. for $C_8H_9N_2O_3S$ (M^+ + H): 213.0334. Found: 213.0349.

Analytical procedure. — *Reagents:* (*1*) periodic acid reagent, 25mM periodic acid in 62.5mM sulfuric acid; (*2*) arsenite solution, 2% sodium arsenite in 0.5M HCl; (*3*) 2-thiobarbituric acid reagent, 6% in water, adjusted to pH 2 with 0.1M NaOH solution. *Method:* An aqueous solution of L-mycarose of proper concentration (0.5–60 μg in a volume of 250 μL) was treated with 30 μL of the periodate reagent for 20 min at 25°. The reaction was then terminated by the addition of 40 μL of the arsenite solution to reduce the excess of periodate. The addition of 200 μL of 2-thiobarbituric acid reagent, followed by heating for 15 min in a boiling-water bath, resulted in the development of a characteristic chromophore. After the solution had been cooled to room temperature, the optical absorbance of the chromogen at 372 nm was read, and calibrated against a blank (without substrate) that was prepared concurrently.

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