

β -D-GLUCOPYRANOSYL-O-L-TYROSINE: SYNTHESIS, PROPERTIES AND TITRE DURING INSECT DEVELOPMENT*

PAO-WEN LU†, KARL J. KRAMER‡¶, PAUL A. SEIB†, DELBERT D. MUELLER‡,
RAAD AHMED§ and THEODORE L. HOPKINS§

†Departments of Grain Science, ‡Biochemistry and §Entomology, Kansas State University, Manhattan, Kansas 66506 and ¶U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502, U.S.A.

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Abstract— β -D-Glucopyranosyl-O-L-tyrosine (tyrosine glucoside) was synthesized by acid-catalyzed fusion of β -glucopyranose pentaacetate with *N*-carbobenzoxy-L-tyrosine methyl ester and subsequent deblocking, transesterification and saponification. The [¹³C]-NMR spectrum was identical to the natural product isolated from the tobacco hornworm, *Manduca sexta* L. This conjugate was detected by HPLC in whole body extracts of the Indian meal moth, *Plodia interpunctella* (Hübner), almond moth, *Ephestia cautella* (Walker) and bagworm, *Thyridopteryx ephemeraeformis* (Haworth). Highest levels were found in mature larvae and pharate pupae. Tyrosine glucoside serves as a primary source of tyrosine and glucose for cuticle formation in Lepidoptera undergoing metamorphosis.

Key Word Index: β -D-Glucopyranosyl-O-L-tyrosine, chemical synthesis, NMR, *Manduca sexta*, *Plodia interpunctella*, *Ephestia cautella*, *Thyridopteryx ephemeraeformis*, haemolymph, whole body, cuticle, Lepidoptera

INTRODUCTION

TYROSINE and glucose are conjugated at specific stages of development by certain insects (CHEN *et al.*, 1978; KRAMER *et al.*, 1980; ISHIZAKI and UMEBACHI, 1980; ISOBE *et al.*, 1981). β -D-Glucopyranosyl-O-L-tyrosine apparently serves as a reservoir of both sugar and amino acid for eventual incorporation into cuticle during metamorphosis. We have synthesized the glucoside and compared its properties to those of the naturally occurring compound isolated previously from the tobacco hornworm, *Manduca sexta* (L.) (KRAMER *et al.*, 1980). We have confirmed the identity of this metabolite and report some of its properties. In addition, a rapid and convenient method for determining its titre in insects is described. The results suggest that this conjugate serves as a source of tyrosine and glucose during larval-pupal metamorphosis of the Indian meal moth, *Plodia interpunctella* (Hübner) and the almond moth, *Ephestia cautella* (Walker).

MATERIALS AND METHODS

Insects

P. interpunctella and *E. cautella* were from stock colonies reared on wheat media at the U.S. Grain Marketing Research Laboratory. Insects were staged by measurement of head capsule size, body weight and degree of cuticle tanning. Last instar bagworm larvae, *Thyridopteryx ephemeraeformis* (Haworth) were field collected.

Extraction and high performance liquid chromatography (HPLC)

Whole insects were homogenized in 0.1 M NaH₂PO₄, pH 3, at a concentration of 100 mg wet weight/ml using a Tekmar[®] tissueizer at 4°C. Homogenates were filtered through a 1.2 μ filter and subjected to HPLC (20 μ l) on a Waters ODS column (3.9 mm i.d. \times 300 mm) using a Waters 6000 pump, Beckman 100-10 variable wavelength detector set at 270 nm and a Waters Data Module printer-plotter. The mobile phase was 0.05 M NaH₂PO₄, pH 3, eluted at 25°C and a flow rate of 1 ml/min. The limit of detection of authentic standard was approximately 100 ng and recovery was \geq 90% from the extractions.

N-Carbobenzoxy-L-tyrosine methyl ester (1)

Carbobenzoylation was accomplished using a minor modification of the procedure described by BERGMANN (1932). L-Tyrosine methyl ester hydrochloride (Aldrich Chemical Co., Inc., Milwaukee, WI, 5 g, 21.6 m-mole) was added to an aqueous solution of 12.5% (w/v) sodium carbonate (20 ml). A mixture of chloroform (30 ml) and acetonitrile (10 ml) was added to the aqueous solution with vigorous stirring for 10 min. If an emulsion formed between the two layers, more acetonitrile was added until the emulsion disappeared. A second portion of 25% (w/v) aqueous sodium carbonate solution (10 ml) was added to the two-phase mixture and carbobenzoxy chloride (3.0 ml)

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was then immediately added dropwise with vigorous stirring and cooling in an ice bath. Thin-layer chromatography of the chloroform layer (40 ml) in the reaction mixture using a mixture of chloroform and ethyl acetate (4:1, v/v) as developing solvent showed that the starting amine (R_f 0.2) had disappeared completely to give the *N*-carbobenzoxy derivative (1) (R_f 0.7). The chromatography was done on plates coated with silica gel; they were visualized by spraying with 50% aqueous sulphuric acid and ashing on a hot plate.

The organic layer was separated and dried over anhydrous sodium sulphate and the chloroform was removed by evaporation using a rotary evaporator. The residue, a white solid, was washed with 30 ml of petroleum ether (b.p. 36–60°C) several times until the smell of carbobenzoxy chloride could not be detected. The solid was dried overnight under vacuum to yield 4.5 g of product with m.p. 93–95°C. PETTIT (1970) reported compound (1) with m.p. 92–93°C.

***N*-Carbobenzoxy-O-(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)-L-tyrosine methyl ester (2)**

N-Carbobenzoxy-L-tyrosine methyl ester (4 g, 11.7 m-mole), β -D-glucose pentaacetate (1 g, 4.6 m-mole, m.p. 132°C, WOLFROM and THOMPSON, 1963) and anhydrous *p*-tolylsulphonic acid (0.1 g) were placed in a round-bottom flask and the mixture heated at ~120°C under vacuum for 15 min. After cooling, the mixture were dissolved in chloroform (30 ml) and thin-layer chromatography using chloroform: ethyl acetate (2:1, v/v) showed that the reaction mixture contained, besides starting materials, a new component with R_f ~ 0.3. The mixture was chromatographed on a column (3.2 cm o.d. \times 60 cm) of silica gel (300 g, 28–200 mesh, Fisher Scientific Company, Fair Lawn, NJ), using a mixture of chloroform and ethyl acetate (2:1, v/v) as developing solvent. The glycoside derivative (2) was eluted as a pure material that crystallized from a mixture of chloroform (5 ml) and petroleum ether (1–2 ml). The white solid (0.75 g, yield 47%) had m.p. 114–116°C and $[\alpha]_D = +24.2^\circ$ (*c* 1.63, CHCl₃). *Anal. Calc.* for C₃₂ H₃₇ N O₁₄: C, 58.27; H, 5.61; and N, 2.12. *Found*: C, 58.21; H, 5.66. U.v. data/(methanol): $\lambda_{max} = 279$ and 272 nm with $\epsilon = 8.37 \times 10^2$ and 9.90×10^2 , respectively.

O-(2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosyl)-L-tyrosine methyl ester (3)

The methyl ester of *N*-carbobenzoxy-O-(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)-L-tyrosine (0.67 g, 1.0 m-mole) was dissolved in glacial acetic acid (14 ml) containing 5% platinum on powdered charcoal (0.1 g). The mixture was stirred under one atmosphere of hydrogen for 3 hr, the catalyst removed by filtration and the filtrate freeze dried to a light brown syrup. Traces of acetic acid were removed from the syrup by repeated addition and evaporation of ethyl acetate. The final product was a syrup (0.51 g, 100% yield), which was homogeneous (R_f 0.9) as determined by paper chromatography. The chromatogram was developed in a descending manner with ethyl acetate, acetic acid and water (6/3/2, by vol) and the spots visualized using ninhydrin-cadmium acetate spray (KRAMER *et al.*, 1980). U.v. spectroscopy of the product in methanol showed two absorption peaks at λ_{max} 268 nm and 274 nm.

(β -D-Glucopyranosyl)-O-L-tyrosine (4)

O-(2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosyl)-L-tyrosine (3) (0.51 g, 1.0 m-mole) was stirred with sodium methoxide (1% in dry methanol, 30 ml) for 1 hr. Methyl acetate was removed by three additions and evaporations of dry methanol (20 ml). After each evaporation step the volume of methanol in the mixture remained at ~10 ml. Distilled water (20 ml) was added and the remaining methanol removed by evaporation. After stirring the aqueous alkaline solution for 1 hr, the mixture was diluted with distilled

water (200 ml) and applied to 50 ml of an anion-exchange resin (AG 1-X8, formate, 200–400 mesh, Bio-Rad Lab., Richmond, CA). The column (2.5 cm i.d. \times 30 cm) was developed with 0.05 M ammonium formate at ~1 ml/min and 5 ml fractions were collected. The desired glycoside was detected by absorbance at 280 nm and eluted as a single component in fractions 14–65. The fractions were combined and evaporated to a semi-solid and the ammonium formate removed by evaporation under vacuum at ~45°C. The residual brown solid was dissolved in warm water (5 ml) and decolorized by addition of charcoal. The clear filtrate was evaporated to a small volume (~3 ml), which yielded white crystals of (β -D-glucopyranosyl)-O-L-tyrosine (4) upon standing at room temperature. After filtration, a second crop of crystals was obtained by cooling to ~4°C. The total yield of the two crops of crystals was 0.2 g (0.6 m-mole, 60% yield). The crystals had m.p. 260–265°C, $[\alpha]_D = 69.43^\circ$ (*c* 0.4, water). *Anal. Calc.* for C₁₅ H₂₁ N O₈: C, 52.48; H, 6.12; and N, 4.08. *Found*: C, 51.70; and H, 6.02. U.v. data (methanol); $\lambda_{max} = 276$ nm and 270 nm, $\epsilon = 7.33 \times 10^2$ and 8.96×10^2 , respectively. β -D-Glucopyranosyl-O-L-tyrosine (4) gave a red-purple spot on paper chromatography when sprayed with ninhydrin-cadmium acetate.

Tyrosine glucoside was isolated from *M. sexta* according to the procedure of KRAMER *et al.* (1980). U.v. data: $\lambda_{max} = 275$ and 269 nm with $\epsilon = 7.40 \times 10^2$ and 6.21×10^2 , respectively in 0.05 M NaH₂PO₄, pH 3. The synthetic glycoside had a mobility identical to the naturally occurring metabolite when analyzed by fingerprint analysis (KRAMER *et al.*, 1980).

NMR spectroscopy

The carbon-13 spectra of the synthetic and naturally occurring glucoside as well as L-tyrosine were measured in H₂O solution at 33°C using external D₂O in a concentric capillary for lock on a Varian XL 100-15 instrument modified by Nicolet Corporation for Fourier transform spectroscopy. Chemical shifts (δ) were measured relative to external sodium 2,2-dimethylsilapentane-2-sulphonate in D₂O or internal dioxane, which resonated at -1.75 and 67.4 ppm respectively, relative to internal TMS.

RESULTS AND DISCUSSION

Synthesis of β -D-glucopyranosyl-O-L-tyrosine

We first attempted to prepare β -D-glucopyranosyl-O-L-tyrosine using the Koenigs-Knoor reaction. When *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Sigma Chemical Co., St. Louis, MO) was reacted with acetobromoglucose and silver oxide in benzene/acetonitrile (2/1, v/v), an undesired product, *p*-nitrophenyl β -D-glucopyranoside, was isolated after deacylation. All other condensation reactions in this work were done using the more stable methyl ester of *N*-carbobenzoxy-L-tyrosine (1). However, no condensation could be promoted between 1 and acetobromoglucose using mercuric cyanide, silver oxide, or a mixture of mercuric bromide and mercuric oxide as catalysts and chloroform or a mixture of benzene and acetonitrile as solvents. It appears the Koenigs-Knoor reaction failed because the phenolic hydroxyl reacted with the various catalysts to give insoluble salts of the amino acid derivative (1).

The Helferich reaction (HELFERICH and SCHMITZ-HELLEBRECHT, 1933), however, gave positive results (Fig. 1). Acid-catalyzed fusion of β -D-glucopyranose pentaacetate with the methyl ester of *N*-carbobenzoxy-L-tyrosine (1) gave the β -glucopyranoside deriva-

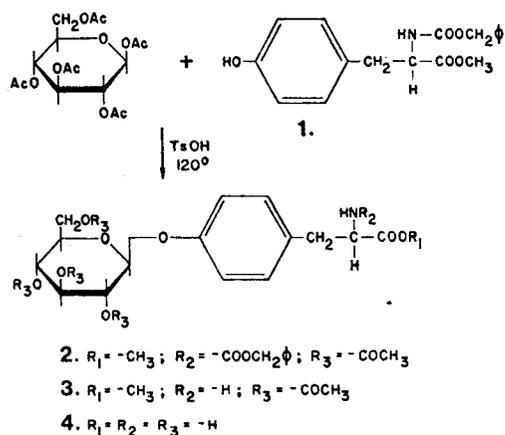


Fig. 1. Chemical synthesis of β -D-glucopyranosyl-O-L-tyrosine (4).

tive (2), which was isolated in crystalline form in 47% yield after silica gel column chromatography.

The carbobenzyoxy group was removed from the blocked glucoside (2) by hydrogenation over platinum on carbon in glacial acetic acid to produce *O*-(2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl)-*O*-L-tyrosine methyl ester (3). Glacial acetic acid was needed as the solvent in the hydrogenation of 2 to prevent the newly formed free amino group from reacting with the ester carbonyl of the amino acid.

Transesterification of compound (3) removed the acetyl blocking groups and saponification hydrolyzed the methyl ester to produce the desired β -D-glucopyranosyl-O-L-tyrosine (4). The glycoside (4) was purified by anion-exchange chromatography to give an analytically pure crystalline solid.

[^{13}C]-NMR

The [^{13}C]-NMR spectrum of the synthetic glycoside was identical to that of the natural product as

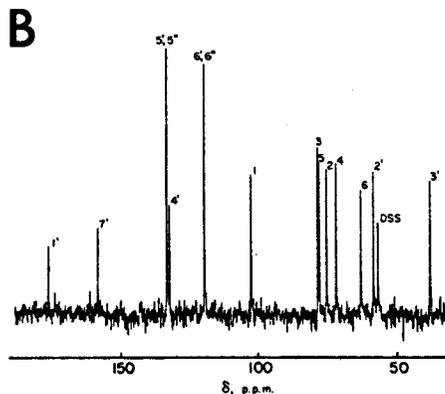
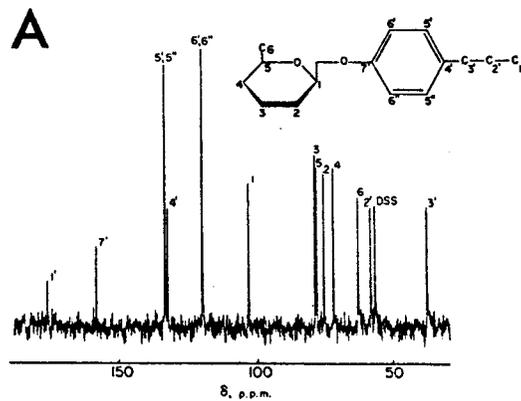


Fig. 2. Carbon-13 NMR spectra of the natural (A) and synthetic (B) β -D-glucopyranosyl-O-L-tyrosine.

shown in Fig. 2. Comparisons of the [^{13}C]chemical shifts for the two β -D-glucopyranosyl-O-L-tyrosines along with those for L-tyrosine and methyl β -D-glucopyranoside are provided in Table 1. The resonances of the L-tyrosinyl carbons in 4 were assigned by first

Table 1. Carbon-13 chemical shifts* of L-tyrosine, methyl β -D-glucopyranoside and β -D-glucopyranosyl-O-L-tyrosine

Carbon Atom	L-Tyrosine δ , ppm			β -D-Glucopyranosyl-O-L-tyrosine δ , ppm [†]	
	pH 0.9	pH 10.8	$\Delta\delta$ [‡]	Synthetic, pH 7	Natural, pH 7
C-1'	172.3	183.2	10.9	174.7	174.7
C-2'	55.2	58.6	3.4	56.9	56.9
C-3'	37.7	40.8	3.1	36.4	36.4
C-4'	126.6	125.8	-0.8	130.6	130.7
C-5'	131.7	131.6	-0.1	131.6	131.6
C-5''					
C-6'	116.9	119.0	2.1	117.8	117.9
C-6''					
C-7'	156.1	163.5	7.4	156.8	156.8
Methyl β -D-glucopyranoside δ , ppm [§]					
C-1		104.2		101.0	101.1
C-2		74.1		73.8	73.8
C-3		76.9		76.4	76.5
C-4		70.7		70.2	70.3
C-5		76.8		77.0	77.0
C-6		61.9		61.4	61.5

* Chemical shifts are relative to internal tetramethylsilane (TMS).

[†] See Fig. 2.

[‡] $\Delta\delta = \delta$ at pH 10.8 - δ at pH 0.9.

[§] ROSENTHAL and FENDLER (1976).

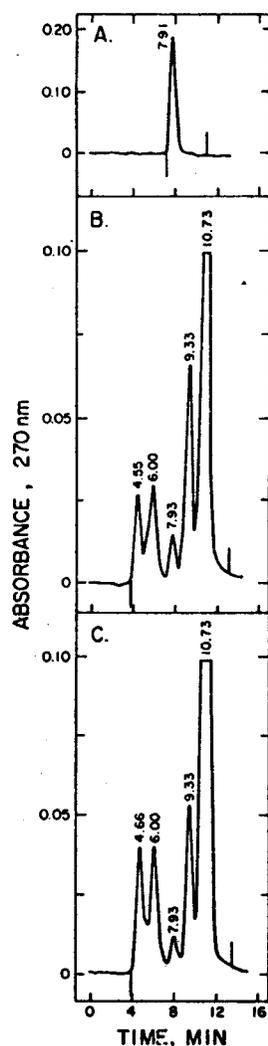


Fig. 3. High performance liquid chromatographic analyses of β -D-glucopyranosyl-O-L-tyrosine with isocratic elution in 0.05 M NaH_2PO_4 , pH 3. A, 20 μl of 7 mM solution of synthetic compound (4). B, 20 μl of 0.1 g/ml extract of *P. interpunctella* last instar larvae. C, Extract of *E. cautella* last instar larvae.

examining the shifts of L-tyrosine at pH 0.9 and 10.8. Ionizations of the carboxylic acid, α -amino and phenolic hydroxyl groups of L-tyrosine permitted identification of the carbon attached to and next to the ionized oxygens. The remaining carbons were readily identified by bond type and ^1H coupling patterns. All the shifts of the L-tyrosinyl carbons in the glycoside at pH 7 were between those of the free amino acid at pH 0.9 and 10.8, except C4'. In the spectrum of the free amino acid, C4' was observed at 126.6 and 125.8 ppm at pH 0.9 and 10.8, respectively, whereas in the glycoside C4' resonated at 130.6 ppm at pH 7. Direct comparison of all the chemical shifts of L-tyrosine to those of the glycoside (4) at pH 7 was not possible, however, because of the extremely limited solubility of the L-tyrosine zwitterion.

The resonances of the carbons on the glucose moiety in 4 also agreed excellently with those observed for methyl β -D-glucopyranoside (ROSENTHAL

and FENDLER, 1976), except for C1 which was no doubt shifted by the aromatic substituent in the glycoside. No trace of α -glucoside was found in the synthetic product since no signal was observed (Fig. 2) at ~ 96 ppm in the anomeric region of the spectrum (STOTHERS, 1972). These results confirm and extend the earlier identification of this metabolite isolated from the tobacco hornworm (KRAMER *et al.*, 1980). In addition, a partial list of ^{13}C chemical shifts for the glycoside from silkworm haemolymph has recently been reported (ISOBE *et al.*, 1981). All of their assignments agreed with ours and the shifts reported were within 0.7 ppm or better of those given in Table 1, except for C2' which differed by 2.1 ppm. Unfortunately, no pH was listed for their solutions and no shifts were given for C1', C4' and C7'.

Titre of β -D-glucopyranosyl-O-L-tyrosine in insect development stages

An HPLC method (Fig. 3A) was developed to assay tyrosine glucoside at various stages of insect development. The retention times for the metabolite and tyrosine on a C_{18} column were 7.9 min and 10.7 min (not shown), respectively. Quantitation was possible after whole insects were homogenized in 0.1 M NaH_2PO_4 , pH 3 and the crude extract passed through a 1.2 μ cellulose filter. Figs 3B and C show an analysis of extracts of mature larvae of *P. interpunctella* and *E. cautella*. The elution profiles were remarkably similar; tyrosine glucoside was well resolved and its titre easily determined. Addition of authentic standard increased the area of the peak at 7.9 min by the expected amount. Fingerprint analysis of the larval metabolite separated by HPLC revealed a single ninhydrin-positive spot with a mobility identical to the synthetic glycoside.

Table 2 shows the results of a study of the temporal relationship between β -D-glucopyranosyl-O-L-tyrosine levels and development. No glycoside was found in eggs or in the early larval instars of the Indian meal moth. During the last larval instar, the glycoside titre increased dramatically. Maximum amounts occurred during pharate pupal development and diminished after the pupa had tanned. We estimate that millimolar levels of tyrosine glucoside are produced in certain insects undergoing metamorphosis. In at least 30 species of Lepidoptera (KRAMER *et al.*, 1980; ISHIZAKI and UMEBACHI, 1980; ISOBE *et al.*, 1981) and in one species of Diptera (CHEN *et al.*, 1978), the conjugate apparently supplies a substantial proportion of the tyrosine from which diphenol substrates are generated for tanning and cross-linking reactions in cuticle.

We have previously determined that tyrosine glucoside synthesis was initiated during the last larval stage but not in the penultimate instar of the tobacco hornworm (KRAMER *et al.*, 1980). The conjugate was also detected in the pupa but not in the adult, suggesting a unique role as a storage form for both the aromatic amino acid moiety used in sclerotization of pupal cuticle and for carbohydrate used in chitin synthesis as suggested by ISOBE *et al.* (1981). The latter study showed that tyrosine glucoside was the major haemolymph sugar during late pupal-adult development and was either absent or at much reduced levels in larvae, tanning pupae and moths of *Bombyx mori*. No tyrosine glucoside was found in silkworm larval hae-

Table 2. Titres of β -D-glucopyranosyl-O-L-tyrosine in developmental stages of *Plodia interpunctella* and *Ephestia cautella*

Species	Stage	Concentration*
<i>P. interpunctella</i>	eggs	<0.1
	immature larva (4th instar)	<0.1
	mature larva (5th instar)	1.1 \pm 0.1
	pharate pupa	3.7 \pm 0.6
	partially tanned pupa	0.4 \pm 0.1
	tanned pupa	<0.1
<i>E. cautella</i>	adult	<0.1
	mature larva (5th instar)	2.1 \pm 0.2
	tanned pupa	<0.1
	adult	<0.1

* mg/g wet wt. \pm S.E.M. $n = 3$.

molymp, but it was detected in larval haemolymph of the armyworm, *Leucania separata*. In the papilionid butterfly, *Papilio xuthus*, tyrosine glucoside was produced at the prepupal stage and in the pupae prior to adult eclosion (ISHIZAKI and UMEBACHI, 1980). The data reported here show that, as was found for *M. sexta* (KRAMER *et al.*, 1980), tyrosine glucoside was synthesized only prior to and during pupal tanning in *P. interpunctella* and *E. cautella*. Apparently there are species variations as to when tyrosine glucoside is synthesized and utilized during insect development.

Earlier we reported that one species of Lepidoptera, the bagworm, *T. ephemeriformis* did not show β -D-glucopyranosyl-O-L-tyrosine using a fingerprint mapping technique (KRAMER *et al.*, 1980). However, these larvae were of unknown age and instar. With the more sensitive HPLC assay, levels ranging from 3–10 mg/g have been measured in mature last stage larvae of that species. Tyrosine glucoside has now been detected in every species of Lepidoptera so far examined and thus appears to be an ubiquitous storage form of phenolic amino acid and glucose evolved by this order of insects.

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