JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

β-D-Glucosyl and α-D-Galactosyl Yariv Reagents: Syntheses from *p*-Nitrophenyl-D-glycosides by Transfer Reduction Using Ammonium Formate

DOMINICK V. BASILE* AND IRAJ GANJIAN

Departments of Biological Sciences and Chemistry, Lehman College of the City University of New York, Bronx, New York 10468

Yariv β-D-glucosyl (**4a**) and Yariv α-D-galactosyl (**4b**) reagents are multivalent phenylglycosides. The β-D-glucosyl reagent is considered diagnostic for arabinogalactan proteins (AGPs) to which it can reversibly bind, stain, and precipitate. The α-D-galactosyl reagent does not bind AGPs and is used as a control. In a new strategy, we accomplished the large scale synthesis of the Yariv reagents in one continuous step by a transfer reduction method and without a need for any specialized apparatus. As the starting material, *p*-nitrophenyl-D-glycosides (**1**) were reduced to *p*-aminophenyl-D-glycosides (**2**) using ammonium formate as the hydrogen donor. The excess formate was converted to formic acid and ammonia, which then were removed from the reaction by simple distillation. Without isolation, *p*-aminophenyl-D-glycosides were diazotized (**3**) and coupled to phloroglucinol to give the Yariv reagents in ∼40% yield. AGPs are a major component of gum arabic, an emulsifying agent widely used in the food and pharmaceutical industries. Increasing interest in AGPs prompted the development of a relatively easy and inexpensive method for the synthesis of these reagents.

KEYWORDS: Yariv reagents; arabinogalactan proteins; ammonium formate; synthesis; transfer reduction

INTRODUCTION

Yariv β-D-glucosyl reagent [1,3,5-tris(4-β-D-glucopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] (**4a**) is a multivalent phenylglucoside (*1*). This reagent is variously used to detect (stain) (*2*−*6*), selectively precipitate (*7*−*9*), and quantify (*10*) macromolecules belonging to a large family of proteoglycans and glycoproteins associated with virtually all plant cell surfaces. Although most of the weight of the glycoproteins is derived from its complex carbohydrate moiety, all members of this family are commonly referred to as arabinogalactan proteins or AGPs. Their ubiquitous presence at plant cell surfaces suggests one or more basic functions. AGPs appear to perform a wide variety of functions in plants including regulation of growth and development and chemoprotection. They are sufficiently variable in composition to serve as determinants of identity at every level of plant organization from cell type to species. Consequently, how AGPs may be functioning to influence the structure and biology of plants has become an intriguing question for an ever-increasing number of investigators (*11*−*15*). How the seemingly infinite variation in composition of individual AGP molecules influences the processing of plant-derived foods and drugs is an area that has been of only limited interest thus far. Gum arabic, derived from *Acacia senegal* Wild., is a commercially important plant product that is known to be rich in AGPs (*16*). The procurement and use of gum arabic in foodstuffs serve as a good example of the value of Yariv β-D-glucosyl reagent in food and agricultural industries. Yariv β-D-glucosyl reagent is used for quality assurance to differentiate between bona fide gum arabic and gums of lesser quality for use in foodstuffs (*17*).

Yariv β-D-glucosyl reagent (**4a**) is considered the most definitive test for detecting the presence of AGPs in plant tissues or plant products (*18*). Because the binding of this reagent is both highly selective and completely reversible, it is proving to be a valuable tool for identifying, quantifying, and affinity selecting/isolating AGPs from aqueous plant extracts (loc. cit.). As the awareness grows that AGPs are always present in plant material but distinctly different in different plant parts and species, there should be an increase in demand for this reagent.

There is at present only one commercial source for Yariv-D-glycosyl reagent (i.e., Biosupplies, Pty. Ltd., Parksville, Victoria, Australia) (*19*). Because of its relatively high cost, however, many workers seek to obtain it as gifts from those who can synthesize it. In an earlier publication, we endeavored to make it easier to prepare β-D-glucosyl Yariv reagent for those investigators with either limited funds or who lacked the training and/or the facilities for organic synthesis (*20*). The procedure described the reduction of *p*-nitrophenyl-β-D-glucoside followed by the diazotization of the corresponding amine and subsequent coupling of the diazonium ion to phloroglucinol. Two methods for the reduction of *p*-nitrophenyl-β-D-glucoside were described as follows: heterogeneous catalytic hydrogenation and catalytic transfer hydrogenation (transfer reduction). The catalytic hy-

* To whom correspondence should be addressed. Tel: 718-960-8800. Fax: 718-960-8236. E-mail: basile@lehman.cuny.edu.

10.1021/jf0401571 CCC: $27.50
Published on Web 11/02/2004

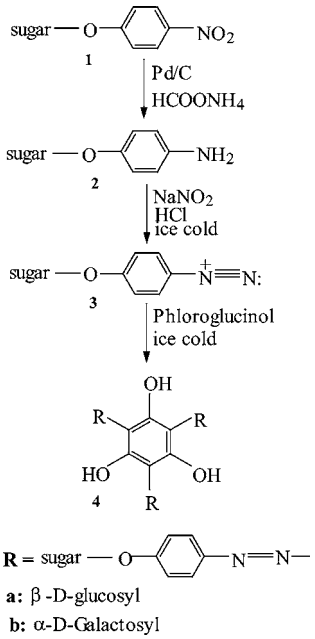

Figure 1. Continuous syntheses of *β*-D-glucosyl (**4a**) and α-D-Galactosyl (**4b**) Yariv reagents from *p*-nitrophenylsugars by transfer reduction.

drogenation method was capable of a lager scale synthesis, of the two methods, but it required the availability of a pressure hydrogenation apparatus. The transfer reduction method described did not require this specialized equipment, but it was limited to small-scale synthesis, due to problems associated with separating the product from an excess of ammonium formate used as the hydrogen donor (*21*). The product, *p*-aminophenyl-*β*-D-glucoside, is soluble in water and could not be extracted with organic solvents. This created a serious problem in removing the excess ammonium formate after the reduction of *p*-nitrophenyl-*β*-D-glucoside (*20*−*22*).

In this paper, we describe a modified transfer reduction method (**Figure 1**) that facilitates a fast removal of ammonium formate, thereby permitting a larger scale synthesis without a need for highly specialized apparatus.

METHODS AND MATERIALS

Chemicals. All reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The water used was purified using a Modulab Analytical Research Grade RO/Polishing water purification system (Continental Water Systems, San Antonio, TX).

Transfer Reduction of *p*-Nitrophenyl-*β*-D-glucoside. To a 500 mL Erlenmeyer flask set on a magnetic stirrer, 250 mL of anhydrous methanol (dried on molecular sieves 3 Å) and 2.5 g (8.3 mmol) of *p*-nitrophenyl-*β*-D-glucoside (**1a**) were introduced. The flask was closed with a rubber septum, and the mixture was stirred for 5 min. The septum was removed, and 0.5 g of 10% Pd/C was added with stirring until the catalyst was fully suspended, followed by the addition of 2.4 g (38.2 mmol) of ammonium formate. The flask was closed, this time with the septum penetrated with a 20-gauge syringe needle (to allow for the release of hydrogen gas) and placed in a water bath set at a temperature of 50 °C, with continuous magnetic stirring. After 20 min, thin-layer chromatography (TLC) of a sample showed a complete conversion of the nitro compound ($R_f = 0.60$) to the amino derivative (**2a**) ($R_f = 0.34$). The reaction mixture was cooled to room temperature and was filtered through a pad of infusorial earth (*23*) (2.5 cm in a 150 mL sintered glass Buchner funnel, packed in methanol). The pad was washed with methanol (100 mL), and the filtrates were combined and evaporated under reduced pressure at 40 °C using a rotary evaporator.

Caution! It is important to add the reagents according to the sequence that they were added. There is a chance of igniting the hydrogen gas (produced in situ) if the catalyst is added after the ammonium formate.

Removal of the Excess Ammonium Formate and Diazotization. Because of high solubility of the amino derivative (**2a**) along with ammonium formate, it was not possible to separate the product from the excess ammonium formate from an aqueous solution by extracting with an organic solvent. Therefore, to remove the excess of the ammonium formate from the reaction, it was converted to formic acid and ammonia by the following process. The residue was dissolved in water (50 mL), and the solution (pH 8.0) was first acidified with 5% v/v sulfuric acid (∼4.1 mL) to pH 3.0. About 25 mL of water was distilled off at 29 °C, under vacuum, to remove the formic acid. It is crucial to keep the distilling temperature below 50 °C, using either a high vacuum distillation set up or a rotary evaporator equipped with vacuum pump. The vacuum generated by a water aspirator is not efficient enough. To remove the ammonia formed in the solution, the volume of the solution was brought back to 50 mL by the addition of water and it was then basified with 5% w/v sodium hydroxide solution (∼6.9 mL) to pH 9.2; then, about 25 mL of the water was distilled off at 29 °C under the high vacuum. Because of the presence of gaseous ammonia, the connection of the flask to the vacuum should be done very gradually and at room temperature using extreme caution in order to avoid an initial splash of the solution that would result from applying a sudden vacuum. After the complete removal of ammonia from the solution of *p*-aminophenyl-*β*-D-glucoside (**2a**), the volume of the solution was brought back again to 50 mL by the addition of water in preparation for the diazotization process. The flask was cooled in an ice bath (ice water, rock salt) and 0.5 M hydrochloric acid (55 mL) was added to the solution. While the solution was kept at a temperature of 0−5 °C, a cold solution of 0.643 g (9.3 mmol) sodium nitrite in water (35 mL) was added dropwise from a separatory funnel over a period of 30 min. To maintain the temperature of the reaction at the 0−5 °C range, small pieces of ice were introduced directly into the reaction flask during the diazotization process, while stirring with a magnetic stirrer.

Coupling of *p*-Diazonium-phenyl-*β*-D-glucoside Ion to the Phloroglucinol. An ice-cold solution of 0.372 g (3.0 mmol) of phloroglucinol in water (120 mL) was added slowly, from a separatory funnel, to the solution of diazonium salt (**3a**), at 0−5 °C, over a period of 30 min while stirring vigorously and maintaining the low temperature. After half an hour, the flask was removed from the ice bath and allowed to warm to room temperature. To the flask containing the deep red solution (pH 2.2), a solution of 0.5% sodium hydroxide (∼44 mL) was added slowly until the pH reached 9.0 and the pH became stable at 9.0 for 1.5 h. The solution (∼300 mL) was diluted with an equal volume of ethanol and was kept overnight in the refrigerator. The precipitate that formed was filtered, and after drying, it resulted in 1.15 g (40%) of the *β*-D-glucosyl Yariv reagent (**4a**).

Preparation of α-D-Galactosyl Yariv Reagent. This compound was synthesized by the same procedure used for the synthesis of *β*-D-glucosyl Yariv reagent described above, with the exception that *p*-nitrophenyl-α-D-galactoside (**1b**) was used as the starting material. The amount of the precipitate, α-D-galactosyl Yariv reagent (**4b**), was 1.10 g (40%).

Caution! In an attempt to collect more product, in both cases, after separation of the first batch, the volume of the supernatant was reduced to 150 mL by evaporation on a rotary evaporator. Further dilution with ethanol (150 mL) and cooling in the refrigerator gave an additional 0.50−0.77 g of a product. The nuclear magnetic resonance (NMR) spectroscopy proved that the second batch was not the Yariv reagent, and it was discarded. After this compound was dissolved in dimethyl sulfoxide (DMSO) (for the NMR), it resulted in a light reddish solution, as compared to the color of a Yariv reagent in DMSO, which was deep red.

ANALYSIS

Verification of products was accomplished by TLC, high-performance liquid chromatography (HPLC), or NMR or some combination of these. TLC was performed using Polygram Sil/G

UV254 plastic sheets (Brinkmann) and a methanol/chloroform 30/70 solvent system. HPLC was carried out using an ISCO model 2360 gradient programmer, a Waters model M 45 solvent delivery system pump, an Altex sample injection valve 210, a Waters model 2487 dual λ UV/vis absorbance detector, and a Kipp and Zonen model BD-41 recorder. The compounds were eluted on a prepacked Beckman Ultrasphere ODS (particle size 5 μm) stainless steel column 25 cm × 4.6 mm i.d. and were detected at 254 nm. The solvent system was methanol/water (50/50, v/v), and the flow rate was 0.5 mL/min. NMR spectra were recorded on a 60 MHz LM 360 Varian instrument. Chemical shifts were reported on the δ scale. The aromatic region (δ 6.0−9.0) of the spectra was used for a diagnostic purpose and verification of the conversion of the nitro compounds to Yariv reagents.

***β*-D-Glucosyl Yariv Reagent.** TLC: *p*-nitrophenyl-β-D-glucoside, $R_f = 0.60$ (**1a**); *p*-aminophenyl-β-D-glucoside (**2a**), $R_f = 0.34$. HPLC: The retention time for *p*-nitrophenyl-β-D-glucoside was 13.5 min, and the retention time for β-D-glucosyl Yariv reagent was 12.0 min. Proton NMR: *p*-nitrophenyl-β-D-glucoside (DMSO) δ 8.1 (d, 2H, $J = 12.0$ Hz), δ 7.1 (d, 2H, $J = 9.0$ Hz); β-D-glucosyl Yariv reagent (**4a**) (DMSO) δ 7.5 (d, 6H, $J = 12.0$ Hz), 7.1 (d, 6H, $J = 12.0$ Hz).

Note. In our previous paper (*20*), the coupling constant (*J*) in the aromatic region for β-D-glucosyl compounds was reported to be 9 Hz and should be corrected to 12 Hz.

α-D-Galactosyl Yariv Reagent. TLC: *p*-nitrophenyl-α-D-galacoside (**1b**), $R_f = 0.66$; *p*-aminophenyl-α-D-galacoside (**2b**), $R_f = 0.40$. HPLC: The retention time for *p*-nitrophenyl-α-D-galacoside was 14.5 min, and the retention time for α-D-galactosyl Yariv reagent was 11.0 min. Proton NMR: *p*-nitrophenyl-α-D-galacoside (DMSO) δ 8.1 (d, 2H, $J = 12.0$ Hz), δ 7.2 (d, 2H, $J = 12.0$ Hz); α-D-galactosyl Yariv reagent (**4b**) (DMSO) δ 7.2 (d, 6H, $J = 12.0$ Hz), 7.6 (d, 6H, $J = 12.0$ Hz).

Caution! The aggregation of the most purified sample of β-D-glucoside Yariv reagent in water has been investigated, using an ultracentrifuge cell at equilibrium (*24*). It has been shown that Yariv compounds undergo a strong self-association in water with a very wide distribution of species. As a result, the usual measurement of physical constants such as melting point, optical rotation, and elemental analysis fail for Yariv compounds. A mixture of disubstituted and trisubstituted phloroglucinol could be formed simultaneously, and also, the aggregating system could readily trap small amounts of the lower homologues, making measurement of the physical constants invalid.

RESULTS AND DISCUSSIONS

The protocol for syntheses of the β-D-glucosyl and α-D-galactosyl Yariv reagents (**Figure 1**; **4a**,**b**) described herein is perceived as an improvement over the two alternate methods described in a previous publication (*20*). This is the first case of using the transfer reduction method for large-scale preparation of Yariv reagents. The synthesis is performed in one continuous synthetic procedure (required time, 2−3 days) without isolating the reaction intermediates, with a good yield (∼40%), using inexpensive starting material (**1a**,**b**). It eliminates the need for access to a pressure hydrogenation apparatus required for the heterogeneous catalytic hydrogenation method. The important change introduced in this revised transfer reduction method was the addition of a two-step process of acidification followed by alkalinization that converts the excess ammonium formate to formic acid and ammonia, respectively. Both of these compounds can be removed easily from the reaction mixture by a simple distillation.

ABBREVIATIONS USED

AGPs, arabinogalactan proteins; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

ACKNOWLEDGMENT

We are grateful to John Klonowsky for his technical assistance. We also thank Yolanda Henry for performing one of the reactions and double checking the procedure.

LITERATURE CITED

(1) Yariv, J.; Rapport, M. M.; Graf, L. The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycoside. *Biochem. J.* **1962**, *85*, 383−388.

(2) Basile, D. V.; Kushner, B. K.; Basile, M. R. A new method for separating and comparing arabinogalactan proteins for the chemosystematics of the *hepaticae*. *Bryologist* **1989**, *92*, 164−169.

(3) Basile, D. V.; Basile, M. R. The occurrence of cell wall-associated arabinogalactan proteins in the *hepaticae*. *Bryologist* **1987**, *90*, 401−404.

(4) Bosch, M.; Knudsen, J. S.; Derksen, J.; Mariani, C. Class III pistil-specific extensin-like proteins from tobacco have characteristics of arabinogalactan proteins. *Plant Physiol.* **2001**, *125*, 2180−2188.

(5) Rauh, R. A.; Basil, D. V. Phenovariation induced in streptocarpus *prolixus* (*Gesneriaceae*) by beta-glucosyl Yariv reagent. *Can. J. Bot.* **2003**, *81* (4), 338−344.

(6) Triplett, B. A.; Timpa, J. D. β-Glucosyl and α-galactosyl Yariv reagents bind to cellulose and other glucans. *J. Agric. Food Chem.* **1997**, *45*, 4650−4654.

(7) Motose, H.; Sugiyama, M.; Fukuda, H. An arabinogalactan protein(s) is a key component of a fraction that mediates local intercellular communication involved in tracheary element differentiation of *zinnia mesophyll* cells. *Plant Cell Physiol.* **2001**, *42*, 129−137.

(8) Classen, B.; Witthohn, B. W. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent. *Carbohydr. Res.* **2000**, *327*, 497−504.

(9) Classen, B.; Blaschek, W. An arabinogalactan-protein from cell culture of *Malva sylvestris*. *Planta Med.* **2002**, *68* (3), 232−236.

(10) Chapman, A.; Helleboid, S.; Blervacq, A. S.; Vasseur, J.; Hilbert, J. L. Removal of the fibrillar network surrounding Cichorium somatic embryos using cytoskeleton inhibitors: Analysis of proteic components. *Plant Sci.* (*Shannon, Ireland*) **2000**, *150*, 103−114.

(11) Evazzadeh, J. A.; Nothnagel, E. A. Cell cycle arrest by perturbation of arabinogalactan- proteins with Yariv phenylglycoside. *Cell Development in Biology. Arabinogalactan-Proteins*, Proceedings of the Symposium on Plant Physiology, 20th 1999; Kluwer Academic/Plenum: New York, NY, 2000; pp 71−81.

(12) Chapman, A.; Helleboid, S.; Blervacq, A. S.; Vasseur, J.; Hilbert, J. L. Arabinogalactan-proteins in Cichorium somatic embryogenesis: Effect of β-glucosyl Yariv reagent and epitope localisation during embryo development. *Planta* **2000**, *211*, 305−314.

(13) Gao, M.; Showalter, A. M. Yariv reagent treatment induces programmed cell death in Arabidopsis cell cultures and implicates arabinogalactan protein involvement. *Plant J.* **1999**, *19*, 321−331.

(14) Thompson, H. J. M.; Knox, J. P. Stage-specific responses of embryogenic carrot cell suspension cultures to arabinogalactan protein-binding β-glucosyl Yariv reagent. *Planta* **1998**, *205*, 32−38.

(15) Basile, D. V.; Basile, M. R.; Mignone, M. M. Arabinogalactan-proteins, place-dependent suppression and plant morphogenesis. *Cell Development in Biology. Arabinogalactan-proteins*, Proceedings of the Symposium on Plant Physiology, 20th 1999; Kluwer Academic/Plenum: New York, NY, 2000; pp 169−178.

(16) Ward, F. M. Uses of gum arabic (*Acacia* sp.) in the food and pharmaceutical industries. *Cell Development in Biology. Arabinogalactan-proteins*, Proceedings of the Symposium on Plant Physiology, 20th 1999; Kluwer Academic/Plenum: New York, NY, 2000; pp 231−139.

(17) Menzies, A. R.; Osman, M. E.; Malik, A. A.; Baldwin, T. C. A comparison of the physicochemical and immunological properties of the plant gum exudates of *Acacia senegal* (gum arabic) and *Acacia seyal* (gum tahla). *Food Addit. Contam.* **1996**, *13*, 991−999.

(18) Nothnagel, E. A. Proteoglycans and related compounds in plant cells. *Int. Rev. Cytol*. **1997**, *174*, 195−291.

(19) Biosupplies Australia, Pty. Ltd., P.O. Box 835 Parksville, Victoria 3052, Australia. Fax: +613-93471071. E-mail: enquiries@biosupplies.com.au. Internet: www.biosupplies.com.au. Check the Internet for a price list. The current prices for 2 mg are $58 for Yariv β-glucosyl reagent and $68 for α-galactosyl Yariv reagent.

(20) Ganjian, I.; Basile, D. V. Reductive syntheses of *p*-aminophenyl-β-D-glucoside and its conversion to β-glucosyl Yariv reagent. *Anal. Biochem.* **1997**, *246*, 152−155.

(21) Roy, R.; Tropper, F. D.; Morrison, T.; Boratynski, J. Syntheses and transformations of glycohydrolase substrates into protein conjugates based on Michael additions. *J. Chem. Soc. Chem. Commun.* **1991**, (7), 536−538.

(22) Roy, R.; Tropper, F. D.; Romanowska, A. New strategy in glycopolymer syntheses. Preparation of antigenic water-soluble poly(acrylamide-*co*-*p*-acrylamidophenyl β-lactoside). *Bioconjugate Chem.* **1992**, *3*, 256−261.

(23) Sigma Aldrich chemical: http://www.sigmaaldrich.com/Brands/Aldrich.html; Kieselguhr (Infusorial Earth) product no. 18514, or Celite 500 fine product no. 22145. It could also be purchased from Fisher Scientific: www. Fishersci.com; as Diatomaceous Earth catalog no. S75114.

(24) Woods, E. F.; Lilley, G. G.; Jermyn, M. A. The self-association of glycosyl phenyl azo dyes (Yariv antigens.). *Aust. J. Chem.* **1978**, *31* (10), 2225−2238.

Received for review March 29, 2004. Revised manuscript received August 28, 2004. Accepted September 10, 2004.

JF0401571