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Chemical synthesis and growth-promoting activity of all-*trans*-retinyl β-D-glucuronide

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All-*trans*-retinol reacts with methyl (2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-β-D-glucopyran)uronate in the presence of Ag_2CO_3 to give the triacetate methyl ester of retinyl β-glucuronide. Hydrolysis of this ester with sodium methylate in methanol gives retinyl β-D-glucuronide in about 15% yield. The water-soluble retinyl β-D-glucuronide was characterized by u.v.–visible, n.m.r. and mass spectra, by elemental analysis and by its susceptibility to hydrolysis by bacterial β-glucuronidase. Retinyl β-glucuronide, when administered intraperitoneally in saline (0.9% NaCl), supports well the growth of vitamin A-deficient rats.

INTRODUCTION

Retinoyl β-glucuronide (I) and retinyl β-glucuronide (II) (Fig. 1) were first identified as prominent biliary metabolites of vitamin A over 20 years ago (Zachman *et al.*, 1966; Lippel & Olson, 1968). Although originally considered primarily as excretion products, they were found to be involved in an enterohepatic circulation (Zachman *et al.*, 1966), and retinoyl glucuronide was shown to stimulate the growth of vitamin A-deficient rats (Nath & Olson, 1967). The more recent observation that retinoyl β-glucuronide is formed in the intestine (Zile *et al.*, 1982; Cullum & Zile, 1985) and corpus luteum (Sklan & Halevy, 1984) as well as in the liver (Zachman *et al.*, 1966) have stimulated renewed interest in their physiological roles. In this regard, all-*trans*-retinoyl β-glucuronide is highly active in stimulating the differentiation of vaginal epithelia (Sietsema & DeLuca, 1982). Furthermore, 13-*cis*-retinoic acid, frequently employed in the clinical treatment of skin disorders, readily forms both 13-*cis*- and all-*trans*-retinoyl β-glucuronide in several tissues (Lippel & Olson, 1968; Zile *et al.*, 1982; McCormick *et al.*, 1983; Meloche & Besner, 1986). Intracellularly, these retinoid β-glucuronides are synthesized by the transfer of the glucuronyl moiety from UDP-glucuronic acid by a glucuronyltransferase present in the microsomes (microsomal fractions) of liver (Lippel & Olson, 1968; Miller & De Luca, 1986) and presumably of other tissues. Interestingly, both retinoid glucuronides

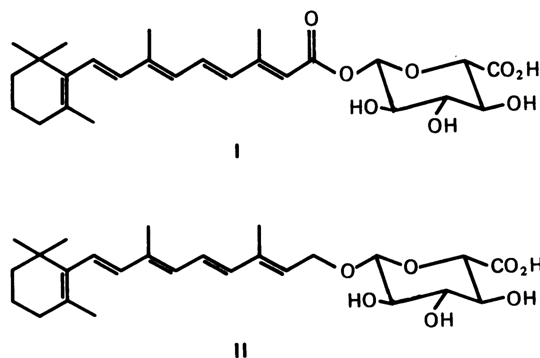

Fig. 1. Structures of all-*trans*-retinoyl β-D-glucuronide (I) and all-*trans*-retinyl β-D-glucuronide (II)

(Barua & Olson, 1986; Barua *et al.*, 1986) and retinoic acid (DeRuyter *et al.*, 1979; Barua & Olson, 1986) are endogenous steady-state components of human blood.

To allow a more detailed study of the actions of these interesting, naturally occurring, water-soluble, metabolites of vitamin A, we previously reported the chemical synthesis and physicochemical characterization of all-*trans*-retinoyl β-glucuronide (Barua & Olson, 1985*a*,*b*) by use of the valuable acylating agent all-*trans*-retinoyl fluoride (Barua & Olson, 1982). In the present paper we report the chemical synthesis, characterization and the growth-promoting activity of all-*trans*-retinyl β-D-glucuronide.

RESULTS AND DISCUSSION

All-*trans*-retinyl β-D-glucuronide was prepared in 15% yield, on the basis of the 1-bromo precursor used, and characterized by chemical, chromatographic and enzymic methods. Properties of the glucuronide are summarized in Table 1.

In regard to its synthesis, when all-*trans*-retinol was stirred with methyl (tri-*O*-acetyl-1-bromoglucopyran)-uronate for 24 h, some retinyl glucuronide was formed, but the yields were very low. We found that refluxing the reactants in a high-boiling-point solvent such as benzene increased not only the rate of glucuronide formation, but also the rate of its destruction. The best results have so far been obtained by refluxing a large excess of retinol with the bromo sugar in diethyl ether for about 3 h. Because the triacetate-methyl ester of retinyl glucuronide and retinol co-migrated together on silica-gel columns, the crude reaction mixture containing the glucuronide and retinol was hydrolysed directly with sodium methylate to give free retinyl glucuronide, which was very strongly adsorbed on silica gel. Unconverted retinol could then be easily separated from retinyl glucuronide by column chromatography. This purified retinyl β-glucuronide was found to be more than 95% pure by h.p.l.c.

The structure of all-*trans*-retinyl β-glucuronide was confirmed by study of its u.v., n.m.r. and mass spectra (not shown here). Its u.v. spectrum, which shows an absorption maximum at 328 nm in water, is expectedly very similar to that of retinol. In the proton n.m.r.

Table 1. Properties of all-*trans*-retinyl β-D-glucuronide

Property	Value or comment
M_r	462 (free acid); 602 (triacetyl methyl ester)
Absorption maxima	325 nm ($A_{1\,cm}$ 973) in methanol and 328 nm ($A_{1\,cm}$ 723) in water
Melting point	135–140 °C (uncorrected, with decomposition)
Elemental composition	$C_{26}H_{38}O_7$, 4.5 H_2O
β-Glucuronidase action	90% Hydrolysis
Chromatographic behaviour	t_R = 1.9 min, as against 11.9 min for retinol on a 5 μm 'Resolve' C_{18} column in methanol/ water (4:1, v/v); flow rate 1.5 ml/min t_R = 17 min on a Partisil 10 ODS-3 column in methanol/ water (4:1, v/v); flow rate 4 ml/min
Solubility	Freely soluble in water, dimethyl sulphoxide and dichloromethane; less so in methanol, ethanol and diethyl ether; very poorly in hexane.

spectrum of all-*trans*-retinyl β-glucuronide, the signals assigned to retinyl protons are almost the same as in retinol, except for the expected absence of a signal for the C-15 hydroxy proton. Signals for the glucuronyl protons (2.9–5.7 p.p.m.) are present mainly as multiplets, even after exchange of the hydroxy protons with deuterium. The tentative assignments of signals given are consequently based primarily on the more-clearly-defined signals of the glucuronyl protons of retinoyl β-glucuronide (Barua & Olson, 1985*b*). An average of 4.5 molecules of water is also associated with retinyl glucuronide, as indicated both by the n.m.r. spectrum and by elemental analysis. Because of the low volatility and instability of retinyl glucuronide, the molecular ion (M_r 462, M^+) was not observed in the mass spectrum of free retinyl glucuronide. However, the molecular ion (M_r 602) could be seen in the mass spectrum of the more stable triacetate methyl ester. Mass fragments characteristic of the retinyl moiety, however, could be seen in both spectra. Further evidence of the structure of retinyl β-glucuronide was obtained by the rapid and essentially complete hydrolysis of the glucuronide to retinol by β-glucuronidase (Fig. 2).

In the rat growth assay, retinyl β-glucuronide was found to be as effective on a molar basis as retinyl acetate, if not more so, in stimulating the growth of depleted rats, but only when injected intraperitoneally (Table 2). In another similar experiment, five vitamin A-deficient female rats, which were losing an average of 22 g/week, gained 22 ± 12 g/week after being injected intraperitoneally at weekly intervals with 404 nmol of retinyl glucuronide in 0.9% NaCl (58 nmol/day) for 3 weeks. In contrast, orally administered retinyl β-glucuronide was essentially inactive (Table 2). Because the retinoid glucuronides are known to be absorbed from the intestine (Zachman *et al.*, 1966), destruction of the orally administered material, which is already quite unstable in aqueous solution, might well be enhanced by exposure to stomach acidity. Further studies on procedures for stabilizing the compound for oral administration clearly are warranted.

EXPERIMENTAL

Chemicals and solvents

Compounds used and their purveyors were: all-*trans*-retinyl acetate and β-glucuronidase from *Escherichia coli*, activity 570000 units/g (Sigma Chemical Co., St. Louis, MO, U.S.A.); D-glucurono-6,3-lactone, hydrobromic acid [30% (w/w) in acetic acid] and 99% Ag_2CO_3 (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); sodium methylate, $HClO_4$ (70%), methanol, dichloromethane, ethyl acetate and hexane (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.)

Chromatographic techniques

Reversed-phase h.p.l.c. was performed by means of a Rheodyne (Cotati, CA, U.S.A.) model 7125 injector, a Waters Associates (Milford, MA, U.S.A.) 6000A pump, an ISCO (Lincoln, NE, U.S.A.) V^4 detector, set at 325 nm, and a Shimadzu (Kyoto, Japan) CR-3A integrator. Methanol/water (4:1 or 17:3, v/v) containing 10 mM-ammonium acetate was run, at a flow rate of 1–4 ml/min, through either a Whatman Lab Sales (Hillsboro, OR, U.S.A.) semi-preparative Partisil 10 ODS-3 column (9.4 mm × 50 cm) or a Waters Associates analytical 'Resolve' 5 μm C_{18} column (3.9 mm × 15 cm). These columns were always preceded by a guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.). Column chromatography was carried out on a specially processed silica gel for dry-column chromatography, namely Activity III/30 (Woelm Pharma, Eschewage, Germany), which was purchased from Universal Scientific (Atlanta, GA, U.S.A.). Silica-gel columns were wet-packed with hexane.

Physicochemical and spectrophotometric analysis

U.v. spectra were recorded with a Shimadzu UV-240 recording spectrophotometer. Mass spectra were obtained with a Finnigan (San José, CA, U.S.A.) model 4000 g.c.–m.s. spectrometer by use of a direct inlet probe and the EI mode for the derivatized retinyl glucuronide, and by use of the desorption chemical-ionization technique with methane as a carrier gas for the non-derivatized retinyl glucuronide. The mass and the relative intensities (in parentheses) of only the major and diagnostic peaks are given. The molecular ion is designated as M^+. The ^{1}H-n.m.r. spectra were recorded by use of a Nicolet Instrument Corp. (Madison, WI, U.S.A.) 300 MHz instrument, with [$^2H_6$]dimethyl sulphoxide as the solvent. Melting points, determined by the open-capillary-tube method, are uncorrected.

Growth assay

Weanling male Sprague–Dawley rats obtained from Holtzman Co. (Madison, WI, U.S.A.) were maintained

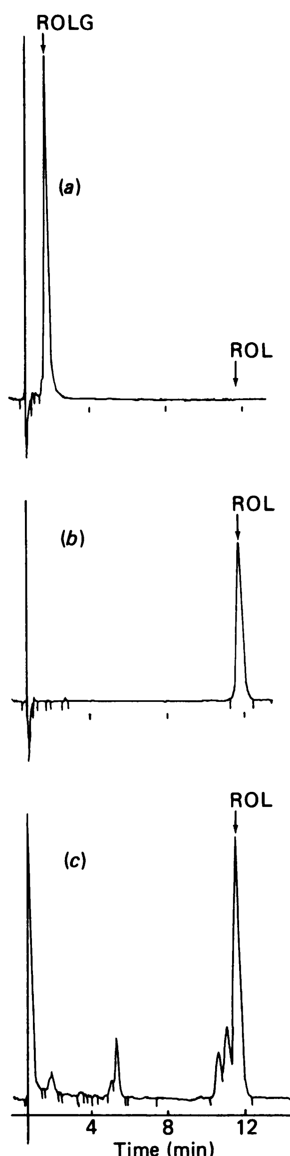

Fig. 2. Enzymic hydrolysis of retinyl β-glucuronide with β-glucuronidase

Chromatogram obtained with reversed-phase h.p.l.c. of: (*a*) all-*trans*-retinyl β-glucuronide (0.1 μg); (*b*) all-*trans*-retinol (0.045 μg); and (*c*) compounds isolated after incubation of all-*trans*-retinyl β-glucuronide with β-glucuronidase at 37° for 2 h. For details, see the Experimental section. Abbreviations: ROLG, retinyl β-glucuronide; ROL, retinol.

on a vitamin A-deficient diet (diet no. 904646; ICN Biochemicals, Cleveland, OH, U.S.A.) for 3 weeks to deplete their reserves of vitamin A, or for 5–6 weeks to make them vitamin A-deficient. The weight of each rat was recorded daily from 1 week before dosing until the end of the experiment. For intraperitoneal administration, 500 μl of an aqueous solution of all-*trans*-retinyl β-glucuronide (0.49 or 0.81 μmol/ml) in 0.15 M-NaCl were injected at weekly intervals. For oral administration, 25 μl of retinyl acetate (0.64 μmol/ml) or of retinyl glucuronide (0.81 μmol/ml) in 90% (v/v) ethanol were given twice daily by use of a positive-displacement micropipette (Gilson Microman, purchased from Rainin Instrument Co., Woburn, MA, U.S.A.).

Synthesis of all-*trans*-retinyl β-D-glucuronide

Methyl (2,3,4-triacetyl-1-bromo-1-deoxy-α-D-glucopyran)uronate was synthesized according to the procedure outlined by Bollenback *et al.* (1955). In brief, glucurono-6,3-lactone was converted into methyl glucopyranuronate by stirring a methanolic suspension with sodium methylate. The methyl ester was acetylated with acetic anhydride in pyridine in the presence of $HClO_4$ as catalyst, and the resulting crystalline methyl (1,2,3,4-tetra-acetylglucopyran)uronate was brominated with HBr/acetic acid to give crystalline methyl (triacetyl-1-bromoglucopyran)uronate.

All-*trans*-retinyl acetate (5 g) was hydrolysed by refluxing for 10 min in methanol (50 ml) in the presence of sodium methylate (1 g). After cooling and adding water to the solution, retinol was extracted with diethyl ether. The extract was washed with water, dried over anhydrous Na_2SO_4, and then evaporatored to dryness in a rotary evaporator.

The oily residue of retinol (4 g) was dissolved in anhydrous diethyl ether (50 ml) to which methyl (triacetyl-1-bromo-1-deoxy-glucopyran)uronate (3 g) and Ag_2CO_3 (2 g) were added. The mixture was refluxed in the dark for 3–4 h. The mixture was then cooled and filtered, and the residue was washed with diethyl ether three times. The pooled filtrate was evaporated to dryness in a rotary evaporator to give a syrupy residue containing the crude triacetate methyl ester of retinyl glucuronide and unconverted starting materials.

The crude product was hydrolysed by refluxing for 10 min in dry methanol (25 ml) containing sodium methylate (1.2 g). The solution was cooled, diluted with water (25 ml), and made slightly acidic with 1 M-HCl. After extraction three times with ethyl acetate (150 ml), the pooled extract was washed with water, dried over

Table 2. Relative growth-promoting activity of all-*trans*-retinyl β-D-glucuronide administered intraperitoneally (I.p.) and orally to vitamin A-depleted rats

Compound administered	Route of administration	*n*	Daily amount (nmol)	Weekly wt. gain* (g)
Retinyl β-glucuronide	I.p.	5	35	37±5
	Oral	3	41	27±6
Retinyl acetate	Oral	5	32	36±10
None	–	4	0	26±10

* Mean ± S.D.

anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The residual oil, dissolved in diethyl ether, was chromatographed on a silica-gel column (2 cm × 30 cm). Development with hexane containing diethyl ether (5–10%, v/v) eluted a yellow zone containing an anhydroretinol-like compound. Unconverted retinol was next eluted with hexane/diethyl ether (1:1, v/v). Upon development with dichloromethane/methanol (9:1, v/v), an unidentified brown side-product was first eluted, followed immediately by retinyl β-glucuronide. Elution of the latter was speeded by use of dichloromethane/methanol (1:1, v/v). All fractions containing retinyl glucuronide, as measured by maximal absorption at 325 nm, were pooled and evaporated to dryness in a rotary evaporator. The resultant pale yellow powder (530 mg, 15% yield on the basis of the 1-bromo precursor) was dissolved in methanol and stored in the dark at −20 °C.

Retinyl β-glucuronide (100 mg) was further purified by h.p.l.c. on the Partisil 10 ODS-3 column by use of methanol/water (4:1, v/v) at a flow rate of 4 ml/min. All-*trans*-retinyl β-glucuronide was eluted in 17 min as the only major peak. Polar impurities constituted less than 5% of the initial mixture.

The triacetate methyl ester of retinyl β-glucuronide was also purified from its crude mixture in methanol by h.p.l.c. on the Partisil 10 ODS-3 column by use of methanol/water (17:3, v/v) at a flow rate of 4 ml/min. Retinol was eluted in 28.6 min and the ester of retinyl glucuronide in 34 min. The fractions containing the ester from several h.p.l.c. runs were pooled, made just acidic with 1 M-acetic acid, and extracted with ethyl acetate. The extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residual oil was dissolved in methanol and stored in the dark at −20 °C.

Physical properties and elemental analysis

The M_r, absorption maxima, melting point and solubility of all-*trans*-retinyl β-glucuronide are summarized in Table 1. Its mass spectrum showed major peaks (*m/z*), with the relative intensities in parentheses, at 285(27), 269(82), 259(47), 249(12), 233(15), 207(3), 193(49), 181(95), 165(34), 257(83), 139(92), 127(100), 117(55), 109(38). N.m.r.: δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 0.99[6H, s, 1-$(CH_3)_2$], 1.43(2H, m, 2-CH_2), 1.56(2H, m, 3-CH_2), 1.66(3H, s, 5-CH_3), 1.79(3H, s, 13-CH_3), 1.90(3H, s, 9-CH_3), 1.98(2H, m, 4-CH_2), 2.90(1H, m, H-2′), 3.09(1H, m, H-3′), 3.15(1H, s, OH-4′), 3.38–3.43(10H, very broad s, H-4′ and 4.5 H_2O), 4.11(2H, d, *J* 6.9 Hz, 15-CH_2), 4.22(1H, m, H-5′), 4.40(1H, m, H-1′), 4.97(1H, broad s, OH-3′), 5.65(1H, t, *J* 7.2 Hz, H-14), 5.74(1H, s, OH-2′), 6.09(1H, d, *J* 15.3 Hz, H-8), 6.13(1H, d, *J* 11.4 Hz, H-10), 6.14(1H, d, *J* 16.8 Hz, H-7), 6.32(1H, d, *J* 15 Hz, H-12), 6.61(1H, dd, *J* 11.4 and 14.7 Hz, H-11). When shaken with ^2H_2O, the singlets at 5.74, 4.97 and 3.15 and the broad peak at 3.38–3.43 disappeared, leaving behind a multiplet at 3.39 (due to H-4′). A strong peak due to $^1HO^2H$ appeared at 3.88 p.p.m. Elemental analysis: found: C, 57.1; H, 8.5; calc. for $C_{26}H_{38}O_7$,4.5 H_2O: C, 57.4; H, 8.6.

The triacetate methyl ester of all-*trans*-retinyl β-glucuronide also showed an absorption maximum of 325 nm in methanol and a mass spectrum with the following peaks (*m/z*): 602 (M^+, 10), 285(7), 269(7), 268(12), 255(11), 220(26), 205(25), 155(14), 73(100). In the presence of sodium methylate the ester was hydrolysed to free retinyl β-glucuronide.

Enzymic conversion of all-*trans*-retinyl β-glucuronide into retinol

All-*trans*-retinyl β-glucuronide (1 μg) was dissolved in water (100 μl) and incubated with or without β-glucuronidase (5 mg ≡ 2850 units) dissolved in 0.1 M-sodium phosphate buffer (2 ml, pH 6.8) at 37 °C for 2 h. The retinoids were extracted with ethyl acetate (3 × 1 ml), the solvent was removed under argon, and the residue was dissolved in methanol (100 μl). Aliquots were injected on to a 5 μm C_{18} column that was developed with methanol/water (4:1, v/v) at a flow rate of 1.5 ml/min. Retinyl β-glucuronide [retention time (t_R) = 1.9 min] was almost completely cleaved to retinol (t_R = 11.9 min) when incubated in the presence, but not in the absence, of the enzyme (Fig. 2). A small unidentified peak of intermediate polarity also appeared at 5.4 min, however, and some *cis* isomers of retinol were also present.

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REFERENCES

Barua, A. B. & Olson, J. A. (1982) Biochim. Biophys. Acta **757**, 288–295

Barua, A. B. & Olson, J. A. (1985*a*) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 772

Barua, A. B. & Olson, J. A. (1985*b*) J. Lipid Res. **26**, 1277–1282

Barua, A. B. & Olson, J. A. (1986) Am. J. Clin. Nutr. **43**, 481–485

Barua, A. B., Batres, R. & Olson, J. A. (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 832

Bollenback, G. N., Long, J. W., Benjamin, D. G. & Lindquist, J. A. (1955) J. Am. Chem. Soc. **77**. 3310–3315

Cullum, M. E. & Zile, M. H. (1985) J. Biol. Chem. **260**, 10590–10596

DeRuyter, M. G., Lambert, W. E. & DeLeenheer, A. P. (1979) Anal. Biochem. **98**, 402–409

Lippel, J. & Olson, J. A. (1968) J. Lipid Res. **9**, 168–175

McCormick, A. M., Kroll, K. D. & Napoli, J. L. (1983) Biochemistry **22**, 3933–3940

Meloche, S. & Besner, J. (1986) Drug. Metab. Dispos. **24**, 246–249

Miller, D. A. & DeLuca, H. F. (1986) Arch. Biochem. Biophys. **244**, 179–186

Nath, K. & Olson, J. A. (1967) J. Nutr. **93**, 461–469

Sietsema, W. K. & DeLuca, H. F. (1982) J. Nutr. **112**, 1481–1489

Sklan, D. & Halevy, O. (1984) Br. J. Nutr. **52**, 107–114

Zachman, R. D., Dunagin, P. E. & Olson, J. A. (1966) J. Lipid Res. **7**, 3–9

Zile, M. H., Inhorn, R. C. & DeLuca, H. F. (1982) J. Biol. Chem. **257**, 3544–3550

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