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(54) Title: C-GLYCOSIDE ANALOGUES OF N-(4-HYDROXYPHENYL)RETINAMIDE-O-GLUCURONIDE

(57) Abstract

The present invention provides breast cancer chemopreventive arylamide analogues of retinoic acid, more particularly C-glycoside analogues of N-(4-hydroxyphenyl)retinamide-O-glucuronide and N-glycoside analogue of retinoyl  $\beta$ -glucuronide that resist both  $\beta$ -glucuronidase mediated enzymatic hydrolysis as well as acid catalyzed hydrolysis. Specifically, the drugs include 4-(retinamido)phenyl-C-glucuronide; 4-(retinamido)phenyl-C-glucoside; 4-(retinamido)benzyl-C-xyloside; 4-(retinamido)benzyl-C-glucoside; 4-(retinamido)benzyl-C-glucuronide; 4-(retinamido)phenyl-C-xyloside, 1-(B-D-glucopyranosyl)retinamide and 1-(D-glucopyranosyluronosyl)retinamide. The invention also relates to a method for making such drugs.

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**C-GLYCOSIDE ANALOGUES  
OF N-(4-HYDROXYPHENYL)RETINAMIDE-O-GLUCURONIDE**

GRANT REFERENCES

This invention was made with government support under grant number CA49837 awarded by the National Cancer Institute. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a continuation-in-part of U.S. Serial No. 07/974,730, filed November 13, 1992.

BACKGROUND OF THE INVENTION

Breast cancer kills thousands of women annually. While surgical intervention has saved the lives of many women, radical and partial mastectomies often prove physically and emotionally debilitating. Indeed, surgery, even when combined with chemotherapy, may still expose the patient to the threat of possible recurrence.

10 A drug that promotes the prevention of breast cancer is desirable, and research efforts have been directed to the development of such drugs. For example, retinoic acid, a vitamin A metabolite, and certain retinoic acid analogues, appear to be necessary for the maintenance of normal epithelial tissue differentiation and can reverse the metaplastic condition of hamster tracheal in vitamin A deficient epithelial tissue. (Newton et al. *Cancer Res.* (1980) 40, 3413-3425).  
15 As a result, retinoic acid and certain amide analogues have been proposed as cancer chemopreventive agents, and display cancer preventive activity (Moon et al. *Cancer Res.* (1979) 39, 1339-1346). Similarly, retinoic acid analogues such as retinyl acetate, 13-*cis*-retinoic acid, glucuronide analogues of retinoic acid also display  
20 cancer preventive activity, including breast cancer preventative activity (Hill, D.L. et al. *Ann. Rev. Nutrition* (1992) 12, 161-181 and Mehta, R.G. et al. *Oncology* (1991) 48, 1505-1509).

25 However, a major impediment to developing retinoic acid and its closely related analogues, has been their relatively high toxicity (Biesalski, H.K. *Toxicology* (1989) 57, 117-161). Side effects such as teratogenicity, hepatotoxicity, scaly skin, hair loss and headaches have been observed as a result of the use of most of these compounds. Researchers have been pursuing the synthesis of retinoic acid analogues with increased potency and/or reduced toxicity for application as  
30 cancer preventative agents. It has been found that N-(4-hydroxyphenyl)

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retinamide (hereinafter "4-HPR") displays chemopreventive activity in breast cancer (Moon et al. *Cancer Res.* (1979) 39, 1339-1346). Indeed, 4-HPR when combined with calcium glucarate, synergistically exerts an increased breast cancer chemopreventive activity in carcinogen-induced rat mammary tumors (Abou-Issa, H.M. et al. *Proc. Natl. Acad. Sci. USA* (1988) 85, 4181-4184). However, the 4-HPR still displays significant cytotoxicity as shown in cultured human breast cancer cells (Bhatnagar, R. et al. *Biochem. Pharmacol.* (1991) 41, 1471-1477).

The glucuronide, N-(4-hydroxyphenyl)retinamide-O-glucuronide (hereinafter "4-HPR-O-glucuronide") has a greater antiproliferative activity and less toxicity in both MCF-7 human mammary cell tumor culture than the 4-HPR. (Bhatnagar, R. et al. *Biochem. Pharmacol.* (1991) 41, 1471-1477.)

However, the 4-HPR-O-glucuronide is unstable; it is hydrolyzed in acidic media and also by the enzyme  $\beta$ -glucuronidase. The propensity to acid hydrolysis may limit the clinical usefulness of 4-HPR-O-glucuronide since oral administration of the drug may reduce the total available concentration of the active drug. Similarly, since  $\beta$ -glucuronidase is present in virtually all mammalian cells, including the blood, liver and intestine, the in vivo half life is quite limited.

It is desirable to have stable chemopreventive drugs, for the prevention and treatment of breast cancer, which resist acid and  $\beta$ -glucuronidase hydrolysis.

#### SUMMARY OF THE INVENTION

The present invention provides breast cancer chemopreventive and treatment arylamide analogues of retinoic acid, more particularly C-glycoside analogues of N-(4-hydroxyphenyl)retinamide-O-glucuronide and N-glycoside analogues of retinoyl  $\beta$ -glucuronide that resist both  $\beta$ -glucuronidase mediated enzymatic hydrolysis as well as acid catalyzed hydrolysis. Specifically, the drugs include 4-(retinamido)phenyl-C-glucuronide; 4-(retinamido)phenyl-C-glucoside; 4-(retinamido)benzyl-C-xyloside; 4-(retinamido)benzyl-C-glucoside; 4-(retinamido)benzyl-C-glucuronide; 4-(retinamido)phenyl-C-xyloside, 1-(B-D-glucopyranosyl) retinamide and 1-(D-glucopyranosyluronosyl) retinamide.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph showing competition for human retinoic acid receptors/BCV between [ $^3$ H]-retinoic acid and: unlabeled retinoic acid, represented by the squares, and 1-(B-D-glucopyranosyl) retinamide, represented by the triangles or 1-(D-glucopyranosyluronosyl) retinamide represented by the diamonds. Values represent the means of three determinations;

Figure 2 is a graph showing competition for human retinoic acid receptors alpha/BCV between [ $^3$ H]-retinoic acid and: unlabeled retinoic

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acid, represented by squares, and 1-(B-D-glucofuranosyl) retinamide, represented by the triangles, or 1-(D-glucofuranosyluronosyl) retinamide represented by the diamonds. Values represent the means of three determinations; and,

5 Figure 3 is a graph showing competition for human retinoic acid receptors beta/BCV between [<sup>3</sup>H]-retinoic acid and: unlabeled retinoic acid, represented by closed squares, and 1-(B-D-glucofuranosyl) retinamide, represented by the triangles, or 1-(D-glucofuranosyluronosyl) retinamide represented by the diamonds. Values represent the means of three determinations.

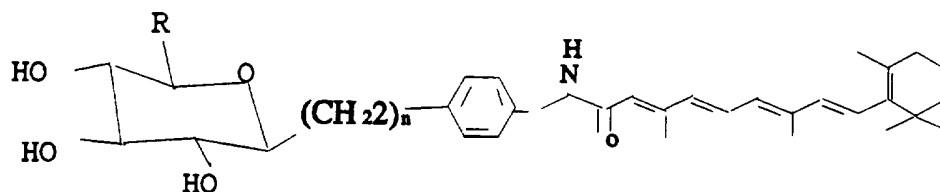
#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides breast cancer chemopreventive drugs that may also be used for the treatment of cancer, that are arylamide analogues of retinoic acid, more particularly C-glycoside analogues of N-(4-hydroxyphenyl)retinamide-O-glucuronide and analogues of retinoyl β-glucuronide that are not hydrolysed by acid nor the enzyme β-glucuronidase. Specifically, the drugs include

15 4-(retinamido)phenyl-C-glucuronide; 4-(retinamido)phenyl-C-glucoside; 4-(retinamido)benzyl-C-xyloside; 4-(retinamido)benzyl-C-glucoside;

20 4-(retinamido)benzyl-C-glucuronide; 4-(retinamido)phenyl-C-xyloside.

The structure of the N-(4-hydroxyphenyl) retinamide-O-glucuronide analogues is represented below:



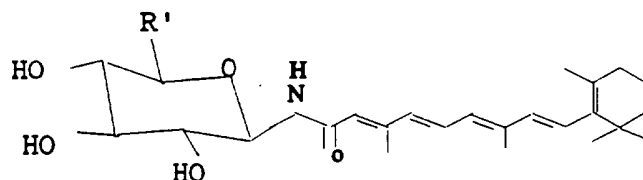
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Wherein: R = COOH, CH<sub>2</sub>OH, or H, and n = 0 or 1.

Where R = COOH and n = 0, the molecule is 4-(retinamido)phenyl-C-glucuronide. Where R = CH<sub>2</sub>OH and n = 0, the molecule is 4-(retinamido)phenyl-C-glucoside. Where R = H and n = 0, the molecule is 4-(retinamido)phenyl-C-xyloside.

Where R = H and n = 1, the molecule is 4-(retinamido)benzyl-C-xyloside. Where R = CH<sub>2</sub>OH and n = 1, the molecule is 4-(retinamido)benzyl-C-glucoside. Where R = COOH and n = 1, the molecule is 4-(retinamido)benzyl-C-glucuronide.

The structure of the retinoyl  $\beta$ -glucuronide analogues is represented below:



wherein R' is H, COOH or CH<sub>2</sub>OH.

Where R = CH<sub>2</sub>OH, the molecule is 1-( $\beta$ -D-glucopyranosyl) retinamide and where R = COOH, the molecule is 1-(D-glucopyranosyluronosyl) retinamide. The drugs may be prepared according to the following examples.

## PREPARATION OF THE DRUGS

Example 1: 4-(Retinamido)phenyl-C-glucuronide

5 First, 14 g of glucose pentaacetate, from Sigma Chemical Company, was dissolved in 60 ml of 30% HBr in acetic acid and allowed to stand a 4° C for 69 hours. The acetic acid was removed. The residue was dissolved in 100 ml of chloroform, washed twice with 100 ml of saturated NaHCO<sub>3</sub>, dried over MgSO<sub>4</sub>, concentrated, and crystallized from 95% ethanol to give 10.88 g (74%) of acetobromoglucose.

10 Next, 5.0 g. acetobromoglucose (12.1 mmol) was dissolved in 125 ml of ether and added over 1 hour to phenylmagnesium bromide prepared from 5.6 g (0.23 g atom, 19 equiv.) of magnesium turnings and 25 ml (237 mmol, 19 equiv.) of bromobenzene in 250 ml of ether. The mixture was heated at reflux for 6 hours then poured into 400 ml H<sub>2</sub>O. The 20 ml glacial acetic acid was added to dissolve the magnesium salts. The mixture was shaken and the layers were separated. The aqueous layer was concentrated to dryness and the residue was reacted with 70 ml of acetic anhydride and 100 ml of pyridine overnight. The mixture was poured into 750 ml H<sub>2</sub>O and the precipitate was isolated by filtration and recrystallized from 2-propanol, to yield 2.236 g (45%), of tetra-O-acetylphenyl-C-glucoside.

20 Next, 1.45g (3.55 mmol) tetra-O-acetyl phenyl-C-glucoside was stirred with 500 mg K<sub>2</sub>CO<sub>3</sub> in 50 ml of methanol overnight. The methanol was removed under reduced pressure and the residue was dissolved in 250 ml H<sub>2</sub>O containing 1 g. of freshly reduced platinum (IV) oxide. Oxygen was bubbled into the solution which was heated to 80°C for 24 hours. The catalyst was removed by filtration and the filtrate concentrated to dryness. The residue was heated to reflux in 200 ml of 1% H<sub>2</sub>SO<sub>4</sub> in methanol for 6 hours. The excess H<sub>2</sub>SO<sub>4</sub> was neutralized with NaHCO<sub>3</sub> and the methanol was removed. The residue was reacted with 70 ml of acetic anhydride and 70 ml of pyridine for 16 hours then poured into 1 L of ice water and the product was isolated by filtration and recrystallized from 2-propanol to yield 724 mg (52%) of methyltri-O-acetyl phenyl-C-glucuronide.

35 Next, 900 mg (2.28 mmol) of methyl tri-O-acetyl phenyl-C-glucuronide was suspended in 20 ml of acetic anhydride and 4.6 g. (19.8 mmol, 8.7 equiv) of copper (II) nitrate was added. The mixture was heated to 100°C for 2 hours then poured into 150 ml ice water. The aqueous phase was extracted twice with 75 ml ether. The combined ether extracts were washed four times with 50 ml saturated NaHCO<sub>3</sub>, dried using MgSO<sub>4</sub>, and the solvent was removed. The product was purified by flash chromatography using 1:1 ethyl acetate/hexanes as eluant to yield 871 mg (87%) of methyl tri-O-acetyl nitrophenyl-C-glucuronide.

40 Next, 1.03 g (2.35 mmol) of the mixture of nitrophenyl-C-glucuronides was added to 100 ml of methanol containing 60 mg of 10%

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palladium on carbon. The mixture was shaken under 40 PSI of hydrogen for 2 hours, then filtered, concentrated, and chromatographed with 1:1 ethyl acetate/hexane as elutant to yield 269 mg (28%) methyl tri-O-acetyl amino phenyl-C-glucuronide.

5 Retinoyl chloride was prepared from 264 mg (0.88 mmol) retinoic acid, and 73  $\mu$ L of pyridine, 65  $\mu$ L (0.88 mmol) of thionyl chloride in 10 ml of ether at 0°C. The mixture was allowed to warm to room temperature over 1 hour. Then 360 mg (0.88 mmol) of 4-(methyltri-O-acetyl) aminophenyl-C-glucuronide was added in 15 ml of benzene  
10 containing 100  $\mu$ L of pyridine. The mixture was stirred at room temperature for 72 hours, diluted with 100 ml ether, and washed once with 50 ml water, then washed twice with 50 ml 0.05 M H<sub>2</sub>SO<sub>4</sub>, and finally washed twice with 50 ml saturated NaHCO<sub>3</sub>. The organic layer was dried using Na<sub>2</sub>SO<sub>4</sub>, concentrated, purified by flash chromatography using 1:1  
15 ethyl acetate/hexanes as elutant to yield 379.5 mg (62%) of 4-(methyltri-O-acetyl) retinamidophenyl-C-glucuronide.

Then 600 mg (0.868 mmol) 4-(methyltri-O-acetyl)retinamidophenyl-C-glucuronide was suspended in 10 ml of 0.2 M sodium methoxide in methanol and stirred at room temperature overnight. To this mixture  
20 was added 600 mg (15 mmol) of sodium hydroxide and 10 ml of H<sub>2</sub>O, which was then dried using Na<sub>2</sub>SO<sub>4</sub>, then concentrated and chromatographed on RP-18 with 85:15 methanol/H<sub>2</sub>O to yield 148 mg (31%) of 4-(retinamido)phenyl-C-glucuronide.

Example 2: 4-(Retinamido)phenyl-C-glucoside

25 First, 14 g of glucose pentaacetate, from Sigma Chemical Company, was converted to 10.88 g of acetobromoglucose as described in Example 1.

Next, 5 g. of the acetobromoglucose (12.1 mmol) was dissolved in 125 ml of ether and added over 1 hour to phenylmagnesium bromide  
30 prepared from 5.6 g (0.23 g atom, 19 equiv.) of magnesium turnings and 25 ml (237 mmol, 19 equiv.) of bromobenzene in 250 ml of ether. The mixture was heated at reflux for 6 hours then poured into 400 ml H<sub>2</sub>O. Then 20 ml glacial acetic acid was added to dissolve the magnesium salts. The mixture was shaken and the layers were separated. The  
35 aqueous layer was concentrated to dryness and the residue was reacted with 70 ml of acetic anhydride and 100 ml of pyridine overnight. The mixture was poured into 750 ml H<sub>2</sub>O and the precipitate was isolated by filtration and recrystallized from 2-propanol, to yield 2.236 g (45%), of tetra-O-acetylphenyl-C-glucoside.

40 Next 5.63 g. (13.8 mmol) tetra-O-acetyl phenyl-C-glucoside was dissolved in 150 ml of acetic anhydride and then 25.58 g (110.4 mmol, 8.0 equiv.) copper (II) nitrate was added. The mixture was heated to 100°C for 1 hour then poured into 400 ml ice water. The organic soluble material was extracted with two ether washes of 200 ml. The

ether extracts were washed twice with 100 ml water and saturated aqueous sodium bicarbonate, dried using MgSO<sub>4</sub>, and concentrated to dryness, to yield 6.11 g (97%) of a 1:1 mixture of o- and p-nitrophenyl-C-glucosides.

5           Next, 1.046 g of the isomeric tetra-O-acetyl nitrophenyl-C-glucoside was dissolved in 150 ml of methanol and about 100 mg of 10% palladium on carbon was added. The mixture was shaken under 40 PSI of hydrogen for 1.5 hours. The catalyst was removed by filtration, the filtrate was concentrated to dryness, and the residue was  
10 chromatographed on silica gel using 1:1 ethyl acetate/hexanes as elutant to give 372.2 mg (38%) of 4-(tetra-O-acetyl) aminophenyl-C-glucoside. A sample of this material having a melting point of 110-111°C was recrystallized from 2-propanol.

          Next, retinoyl chloride was prepared by dissolving 192 mg (0.64  
15 mmol) of retinoic acid in 10 ml of ether containing 100 ml of pyridine and cooled to 0°C. Then 47 µL (0.64 mmol) thionyl chloride was added. The mixture was allowed to come to room temperature over 90 minutes and then 270 mg (0.64 mmol) of the 4-(tetra-O-acetyl) aminophenyl-C-glucoside was added in 9 ml benzene and 1 ml pyridine. The mixture was  
20 stirred at room temperature for 40 hours then diluted with 100 ml ethyl acetate and extracted with 100 ml H<sub>2</sub>O, followed by two washes with 100 ml 1% H<sub>2</sub>SO<sub>4</sub>, and 2 washes with 100 ml saturated aqueous sodium bicarbonate solution. The organic phase was dried using MgSO<sub>4</sub>, concentrated, and chromatographed with 1:1 ethyl acetate/hexanes as  
25 elutant to yield 247 mg (54%) of the 4(tetra-O-acetyl) retinamidophenyl-C-glucoside.

          Next, 247 mg (0.35 mmol) 4-(retinamido)phenyl-C-glucoside tetraacetate was dissolved in 20 ml methanol and 0.2 g of potassium carbonate was added. The mixture was stirred at room temperature for  
30 18 hours, concentrated and chromatographed on RP-18 with 1:1 methanol/water then 3:1 methanol/water then 100% methanol, to yield 47.7 mg (25%) of 4-(retinamido)phenyl-C-glucoside.

Example 3: 4-(Retinamido)phenyl-C-xyloside

          First, 50 g of xylose, from Sigma Chemical Company, 12.5 g of  
35 sodium acetate (anhydrous) and 200 ml of acetic anhydride were heated to reflux for 1 hour and poured into 1 L of ice water. Xylose tetraacetate was isolated by filtration and recrystallized from 95% ethanol to yield 56.75 g (54%).

          Next, 5 g. (15.7 mmol) of the xylose tetraacetate was dissolved  
40 in 200 ml dry chloroform and 8.4 g. (62.8 mmol, 4 equiv), aluminum chloride was added. The mixture was stirred at room temperature for 2 hours, diluted with 200 ml H<sub>2</sub>O, shaken, and separated. The chloroform layer was washed with 200 ml H<sub>2</sub>O, then washed twice with 200 ml saturated aqueous NaHCO<sub>3</sub>, dried using MgSO<sub>4</sub>, and concentrated, to

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provide crude acetochloroxylose. The crude acetochloroxylose was dissolved in 100 ml ether and added to a refluxing solution of phenylmagnesium bromide prepared from 18 ml (169 mmol, 10.8 equiv.) of bromobenzene and 4.12 g (0.169 g atom, 10.8 equiv) of magnesium turnings in 250 ml of ether. The mixture was heated at reflux for 5 hours and poured into 150 ml H<sub>2</sub>O containing 15 ml of glacial acetic acid. The aqueous layer was concentrated to dryness and the residue was reacted with 75 ml acetic anhydride and 75 ml of pyridine for 20 hours. The mixture was poured into 300 ml H<sub>2</sub>O. The product was isolated by filtration and recrystallized from 2-propanol, to yield 1.28 g (24%), tri-O-acetylphenyl-C-xyloside having a melting point of 161-162°C.

Then 1200 mg (3.54 mmol) of the tri-O-acetyl phenyl-C-xyloside was dissolved in 50 ml of acetic anhydride and 6.65 g (28.6 mmol, 8 equiv.) copper (II) nitrate was added. The mixture was heated to 100°C. for 1 hour and then poured into 300 ml of ice water. The water was extracted with 2 washes of 150 ml with ether. The ether extracts were washed twice with 150 ml with water then washed twice with 50 ml saturated NaHCO<sub>3</sub>, then washed with 50 ml brine, dried using MgSO<sub>4</sub>, and concentrated to yield 1.347 g (99%) of a 1:1 mixture of o- and p-tri-O-acetyl nitrophenyl-C-xylosides.

Next, 1.35 g. (3.54 mmol) of the mixture of nitrophenyl-C-xylosides was suspended in 50 ml of methanol and 100 mg of 10% palladium on carbon was added. The mixture was shaken under 40 PSI of hydrogen for 1.5 hours. The catalyst was removed by filtration, and the solvent was removed. The residue was purified by flash chromatography using 1:1 ethyl acetate/hexanes as elutant to yield 375 mg (30%) of 4-(tri-O-acetyl) aminophenyl-C-xyloside.

Retinoyl chloride was prepared by dissolving 320 mg (1.07 mmol) retinoic acid suspended in 10 ml ether containing 200 μl of pyridine. The mixture was cooled to 0°C. and 77 μl (1.07 mmol) of thionyl chloride was added. The mixture was allowed to warm to room temperature over 1.5 hours. The acetylated aminophenyl-C-xyloside was suspended in 16 ml benzene and 800 μl pyridine was added. The mixture was stirred at room temperature for 24 hours, diluted with 25 ml of ethyl acetate, and washed three times with 50 ml water, twice with 25 ml with 5% HCl, and twice with 25 ml with saturated NaHCO<sub>3</sub>, dried using MgSO<sub>4</sub>, and concentrated. The residue was purified by chromatography using 1:1 ethyl acetate/hexanes as elutant to yield 500 mg (74%) of 4-(tri-O-acetyl) retinamidophenyl-C-xyloside having a melting point 96-98°C.

Then 480 mg (0.76 mmol) acetylated retinamidophenyl-C-xyloside was suspended in 10 ml of methanol and 100 mg (0.72 mmol) of potassium carbonate was added. The mixture was stirred at room temperature

overnight, the solvent was removed, and the residue was partitioned between 50 ml ethyl acetate and 50 ml water. The mixture was shaken and the layers separated. The organic layer was dried using  $\text{Na}_2\text{SO}_4$  and the solvent removed. This step yielded 293.5 mg (76%), 4-  
5 (retinamido)phenyl-C-xyloside, having melting point 198-200°C which was used without further purification.

Example 4: 4-(Retinamido)benzyl-C-glucuronide

First, 59 g (109 mmol) of 2,3,4,6-tetra-O-benzyl glucose, from Sigma Chemical Company, was dissolved in 200 ml of pyridine and 22.3 g  
10 (120 mmol) of p-nitrobenzoyl chloride was added. The mixture was stirred at room temperature for 72 hours, poured into 750 ml of water, and the solid was isolated by filtration and recrystallized from 95% ethanol to produce 68.8 g (91%) of 2, 3, 4, 6-tetra-O-benzyl-1-p-nitrobenzoyl glucose.

15 Next, 2.00 g (2.9 mmol) of the 2,3,4,6-tetra-O-benzyl-1-p-nitrobenzoyl glucose was dissolved in 20 ml of dichloromethane and hydrogen bromide was bubbled in for 10 minutes. The p-nitrobenzoic acid was removed by filtration and the filtrate was concentrated to give the crude glycosyl bromide which was dissolved in 50 ml of ether  
20 and added to benzylmagnesium chloride prepared from 600 mg (0.0247 g-atom) of magnesium and 2.8 ml (24.3 mmol) benzyl chloride. The mixture was heated at reflux for 4 hours then poured into 100 ml of water containing 5 ml of acetic acid. The mixture was shaken and separated. The ether layer was washed twice with 50 ml saturated  $\text{NaHCO}_3$ , once with  
25 50 ml brine, dried using  $\text{MgSO}_4$ , and concentrated. The residue was dissolved in 80 ml glacial acetic acid containing 0.1 g of 10% palladium on carbon and shaken under 40 PSI of hydrogen for 19 hours. The catalyst was removed by filtration and the filtrate was concentrated to dryness. The residue was acetylated with 30 ml of  
30 pyridine and 30 ml of acetic anhydride over 16 hours. The reaction was poured into 100 ml of water and extracted twice with 50 ml of ether. The ether extracts were washed twice with 50 ml water, once with 50 ml 5%  $\text{HCl}$ , once with 50 ml saturated  $\text{NaHCO}_3$ , once with 50 ml brine. The extracts were dried using  $\text{MgSO}_4$ , concentrated, and crystallized from 2-propanol to yield 194 mg (16%), tetra-O-acetyl benzyl-C-glucoside  
35 having a melting point of 118-119°C.

Next, 1.56 g (3.70 mmol) tetra-O-acetyl benzyl-C-glucoside was dissolved in 30 ml of acetic anhydride and 7.3 g (31.4 mmol, 8.5 equiv) copper (II) nitrate was added. The mixture was heated to 100°C for 1  
40 hour and poured into 150 ml of ice water. The water was extracted twice with 60 ml of ether. The ether extracts were washed twice with 50 ml water, twice with 50 ml saturated  $\text{NaHCO}_3$ , dried using  $\text{MgSO}_4$ , and concentrated. The crude nitrobenzyl-C-glucoside was dissolved in 30 ml of methanol and 340 mg of potassium carbonate was added. The mixture

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was stirred at room temperature overnight, concentrated, and dissolved in 100 ml of water containing 1 g of freshly reduced platinum (IV) oxide. The mixture was heated to 80°C and oxygen was bubbled in for 10 hours. The catalyst was removed by filtration and the filtrate concentrated to dryness. The residue was heated to reflux in 100 ml of 2% H<sub>2</sub>SO<sub>4</sub> in methanol for 1 hour. Excess H<sub>2</sub>SO<sub>4</sub> was neutralized with 10 ml of pyridine. The mixture was concentrated to dryness and the residue was reacted with 30 ml of pyridine and 60 ml of acetic anhydride for 16 hours. The mixture was poured into 300 ml of ice water and extracted twice with 100 ml ether. The ether layers were washed twice with 200 ml with water, once with 100 ml saturated NaCHO<sub>3</sub>, dried using MgSO<sub>4</sub>, and concentrated to yield 1.107 g (66%) of a 1 to 1 mixture ortho and para methyl tri-O-acetyl nitrobenzyl-C-glucuronides.

The 1.107 g (2.44 mmol) of the 4-nitrobenzyl-C-glucuronide mixture was dissolved in 100 ml of methanol and 100 mg of 10% palladium on carbon was added. The mixture was shaken under 40 PSI of hydrogen for 1 hour, the catalyst was removed by filtration, and the filtrate was concentrated and chromatographed on silica gel using 1:1 ethyl acetate/hexanes as elutant to yield 310 mg (30%) of methyl tri-O-acetyl-4-aminobenzyl-C-glucuronide.

The retinoylated benzyl-C-glucuronide was prepared as in Example 1. From 219 mg (0.73 mmol) of retinoic acid and 310 mg (0.73 mmol) of the protected 4-aminobenzyl-C-glucuronide, 303 mg (59%) of the protected retinamidobenzyl-C-glucuronide having a melting point 80-82°C was obtained after chromatography.

Next, 200 mg (0.28 mmol) of the protect retinamidobenzyl-C-glucuronide was dissolved in 5 ml MeOH and 33 mg sodium methoxide was added. After stirring at room temperature for 16 hours, 5 ml of water and 25 mg of sodium hydroxide were added. The mixture was stirred for another 5 hours, acidified to pH=1 with 5% HCl, and extracted five times with 25 ml ethyl acetate. The ethyl acetate extracts were combined and washed twice with 100 ml water, twice with 50 ml brine, dried using Na<sub>2</sub>SO<sub>4</sub>, and concentrated to yield 74.5 mg (47%) 4-(retinamido)benzyl-C-glucuronide having a melting point of 98-100°C.

35 Example 5: 4-(Retinamido)benzyl-C-glucoside

First, 500 mg. (1.18 mmol) of tetra-O-acetyl benzyl-C-glucoside from Example 4, was dissolved in 10 ml of acetic anhydride and 2.2 g (9.46 mmol, 8 equiv) copper (II) nitrate was added. The mixture was heated to 100°C for 1 hour and poured into 75 ml of water. The water was extracted twice with 40 ml of ether. The ether extracts were washed twice with 40 ml water, twice with 40 ml saturated NaHCO<sub>3</sub>, once with 40 ml brine, then dried using MgSO<sub>4</sub>, and concentrated. The crude nitro benzyl-C-glucoside was dissolved in 25 ml of methanol containing 50 mg of 10% palladium on carbon and was shaken under 40 PSI of

hydrogen for 1 hour. The catalyst was removed by filtration. The filtrate was concentrated and chromatographed on silica gel using 1:1 ethyl acetate/hexanes as elutant, to yield 173.2 mg (33%), 4-(tetra-O-acetyl) aminobenzyl-C-glucoside having a melting point of 145-146°C.

5 The retinamide was prepared as in Example 2. From 108 mg (0.36 mmol) retinoic acid and 158 mg (0.36 mmol) acetylated aminobenzyl-C-glucoside, 133 mg (51%) of 4-(tetra-O-acetyl) retinamidobenzyl-C-glucoside was obtained.

10 Next, 130 mg (0.18 mmol) of acetylated retinamidobenzyl-C-glucoside was dissolved in 8 ml of methanol containing 100 mg of potassium carbonate. The mixture was stirred at room temperature for 16 hours, concentrated, and partitioned between 40 ml of ethyl acetate and 40 ml of water. The mixture was shaken and separated and the ethyl acetate layer was washed twice with 40 ml water, twice with 40 ml  
15 brine, dried using  $\text{Na}_2\text{SO}_4$ , and concentrated, to yield 68 mg (68%) 4-(retinamido)benzyl-C-glucoside.

Example 6: 4-(Retinamido)benzyl-C-xyloside

First, 50 g of xylose, from Sigma Chemical Company, was converted to xylose tetracetate as described in Example 3.

20 Next, 2.07g (6.5 mmol) of the xylose tetracetate was dissolved in 125 ml dry chloroform and 3.3 g (24.8 mmol, 3.8 equiv.) aluminum chloride was added. The mixture was stirred at room temperature for 1.5 hours then washed once with 50 ml saturated  $\text{NaHCO}_3$ , once with 50 ml water, and once with 50 ml brine, then dried using  $\text{MgSO}_4$ , and the  
25 solvent removed. The residue was dissolved in 100 ml ether and added to a refluxing solution of benzylmagnesium chloride prepared from 11.5 ml (0.10 mol) of benzyl chloride and 2.43 g (0.1 g-atom) of magnesium in 250 ml of ether. The mixture was kept at reflux for 4 hours then poured into 400 ml  $\text{H}_2\text{O}$  containing 20 ml acetic acid. The aqueous layer  
30 was concentrated to dryness and the residue reacted with 75 ml acetic anhydride and 75 ml pyridine for 8 hours. The mixture was poured into 400 ml  $\text{H}_2\text{O}$  and extracted three times with 160 ml ether. The ether extracts were washed three times with 100 ml saturated  $\text{NaHCO}_3$ , dried using  $\text{MgSO}_4$ , concentrated, and recrystallized from 2-propanol, to yield  
35 989 mg (43%) tri-O-acetyl benzyl-C-xylopyranoside having a melting point of 118-119°C.

Next, 989 mg (2.82 mmol) of tri-O-acetyl benzyl-C-xyloside was dissolved in 25 ml of acetic anhydride and 4.08 g (17.52 mmol, 62 eq) of copper (II) nitrate was added. The mixture was heated to 100°C for  
40 2 hours and poured into 125 ml of water. The aqueous layer was extracted three times 25 ml with ether and the combined ether extracts were washed six times with 25 ml saturated  $\text{NaHCO}_3$ , dried using  $\text{MgSO}_4$ , and concentrated. This crude material was suspended in 100 ml of methanol containing 100 mg of 10% palladium on carbon. The mixture was

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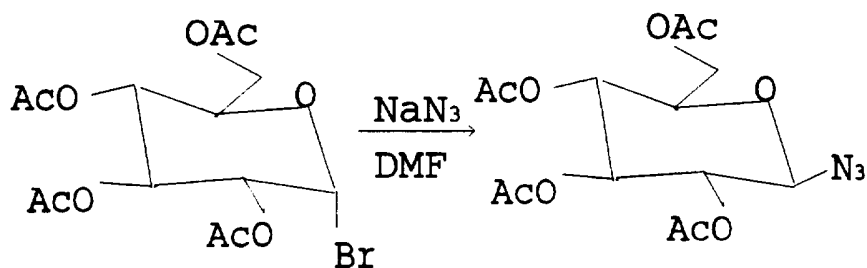
shaken under 40 PSI of hydrogen for 1.5 hours, filtered, and solvent removed. The residue was chromatographed using 1:1 ethyl acetate/hexanes as elutant, to yield 160 mg (16%) of a 4-(tri-O-acetyl) amino benzyl-C-xyloside.

- 5 Retinoyl chloride was prepared in 10 ml of ether from 131 mg (0.44 mmol) retinoic acid, 60 ul pyridine, and 32 ul of thionyl chloride were stirred at 0°C for 1 hour then allowed to warm to room temperature over 30 minutes. Then, pyridine 40  $\mu$ l, 160 mg (0.44 mmol) 4-(tri-O-acetyl) aminobenzyl-C-xyloside, and 10 ml benzene were added.
- 10 The mixture was stirred at room temperature for 72 hours and diluted with 50 ml of ether. The organic extracts were washed once with 50 ml water, twice with 50 ml of 1% H<sub>2</sub>SO<sub>4</sub>, three times with 50 ml saturated NaHCO<sub>3</sub>, once with 50 ml brine, dried using MgSO<sub>4</sub>, and concentrated. The residue was purified by chromatography using 1:1 ethyl acetate/hexanes
- 15 as elutant, to yield 194.5 mg (70%), 4 (tetra-O-acetyl) retinamidobenzyl-C-xyloside having a melting point of 88-90°C.

- Then 180 mg (0.286 mmol) acetylated retinamidobenzyl-C-xyloside was dissolved in 10 ml of methanol containing 150 mg of potassium carbonate. The mixture was stirred at room temperature overnight,
- 20 concentrated, and purified on RP-18 reversed phase silica gel with 100% methanol as elutant, to yield 826 mg (55%) 4-(retinamido)benzyl-C-xyloside.

- In the above examples, the product from multiple runs of a synthesis step may have been pooled to obtain the quantities used in
- 25 subsequent steps.

Example 7: preparation of 1-( $\beta$ -D-glucopyranosyl) retinamide  
Preparation of (2,3,4,6-tetra-O-acetylglucopyranosyl azide)



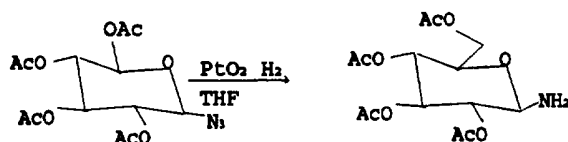
2,3,4,6-tetra-O-  
acetylglucopyranosyl bromide

2,3,4,6-tetra-O-  
acetylglucopyranosyl azide

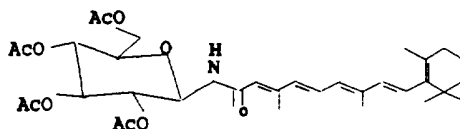
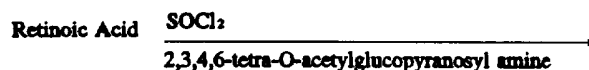
5 First, 2,3,4,6-tetra-O-acetylglucopyranosyl bromide was prepared from glucose pentaacetate by dissolving 50 gm of glucose pentaacetate, available from Sigma Chemical Company, in 200 mL of 30% HBr/acetic acid; the mixture was allowed to stand overnight in the refrigerator. The solvent was removed under reduced pressure, the residue dissolved in CHCl<sub>3</sub>, and the CHCl<sub>3</sub> solution was washed with H<sub>2</sub>O, saturated NaHCO<sub>3</sub>, saturated NaCl, and dried over Na<sub>2</sub>SO<sub>4</sub>. The drying agent was removed by 10 filtration and the residue crystallized from ethanol. The acetobromoglucose has a melting point of 89°C.

Next, 8.22g, that is 20 mmol, of the 2,3,4,6-tetra-O-acetylglucopyranosyl bromide was dissolved in 250 ml of dimethylformamide and 2.6 g, that is, 40 mmol, or two equivalents of 15 sodium azide was added. The mixture was stirred at room temperature for 24 hours and then poured into 500 ml of water. The organic soluble material was extracted 3 times with 150 ml ethyl acetate. The ethyl acetate extracts were washed twice with 100 ml water, then washed with saturated aqueous NaCl (brine), dried over MgSO<sub>4</sub>, and concentrated to 20 dryness. The residue was recrystallized from ethanol to yield 6.1 g, that is, 82% of 2,3,4,6-tetra-O-acetylglucopyranosyl azide. The 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl azide had the following characteristics: melting point 127-129°C; IR (KBr) 2969, 2956, 2118, 1755, 1733, 1373, 1240, 1213, 1072, 1059, 1037; <sup>1</sup>H NMR: δ 1.98 (s, 3H), 2.00 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H), 3.73-3.80 (m, 1H), 4.13 (dd, 1H, J=12.5, 2.4 Hz), 4.25 (dd, 1H, J=12.5, 4.7 Hz), 4.62 (d, 1H, J=8.7 Hz), 4.93 (t, 1H, J=9.26 Hz), 5.07 (t, 1H, J=9.5 Hz), 5.19 (t, 1H, J=9.4 Hz); 25

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Preparation of 2,3,4,6-tetra-O-acetylglucopyranosyl amine2,3,4,6-tetra-O-  
acetylglucopyranosyl azide2,3,4,6-tetra-O-  
acetylglucopyranosyl amine

- 5 4g, that is, 11 mmol of the 2,3,4,6-tetra-O-acetylglucopyranosyl azide was added to 50 ml of dry tetrahydrofuran containing 200 mg of PtO<sub>2</sub>. The mixture was shaken under 40 psi of hydrogen for 2 hours, filtered, concentrated to dryness, to produce 2,3,4,6-tetra-O-acetylglucopyranosyl amine.

10 Preparation of 2,3,4,6-tetra-O-acetylglucopyranosyl retinamide

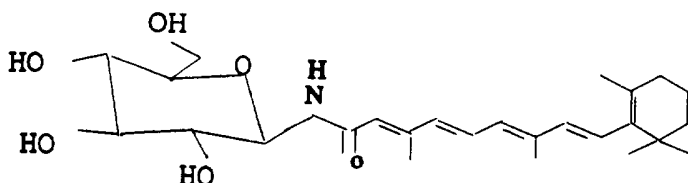
2,3,4,6-tetra-O-acetylglucopyranosyl retinamide

- 15 Retinoyl chloride was prepared from 3.24 g (10.8 mmol) retinoic acid, 1.1 ml of pyridine, and 0.79 ml (10.8 mmol) of thionyl chloride in 60 ml of ether at 0°C. The mixture was allowed to warm to room temperature over 1 hour, and 3.82g, 11 mmol of 2,3,4,6-tetra-O-acetylglucopyranosyl amine was added in 50 ml of benzene containing 3 ml of pyridine. The mixture was stirred at room temperature for 72 hours, diluted with 200 ml of ethyl acetate, and extracted three times

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with 150 ml H<sub>2</sub>O and brine. The organic product was dried over MgSO<sub>4</sub>, concentrated, treated with diazomethane, and chromatographed with 40% ethyl acetate in hexane to remove traces of methyl retinoate, and yielded 4.36 g that is 64% of 2,3,4,6-tetra-O-acetylglucopyranosyl retinamide.

Preparation of 1-(β-D-glucopyranosyl) retinamide



1-(β-D-glucopyranosyl) retinamide

Next, 2.52 g, 4 mmol of 2,3,4,6-tetra-O-acetylglucopyranosyl retinamide was dissolved in 50 ml of methanol and 0.2 g of K<sub>2</sub>CO<sub>3</sub> was added. The mixture was stirred at room temperature for 6 hours, filtered, and concentrated to yield 1.66 g of 1-(β-D-glucopyranosyl) retinamide (90%). The 1-(β-D-glucopyranosyl) retinamide was purified by chromatography on RP-18 with 80% MeOH/H<sub>2</sub>O.

1-(β-D-glucopyranosyl) retinamide has the following characteristics: melting point of 166°C (decomposition); IR (KBr) 3369, 2927, 1662 cm<sup>-1</sup>; UV (CH<sub>3</sub>OH) λ<sub>max</sub> 349 nm (ε 24043); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 1.00 (s, 6H, C(CH<sub>3</sub>)<sub>2</sub>), 1.42 (m, 2H, retinoid H-2)<sup>11</sup>, 1.55 (m, 2H, retinoid H-3), 1.70 (s, 3H, retinoid 5-CH<sub>3</sub>), 1.95 (br s, 5H, retinoid 9CH<sub>3</sub> and H-4), 2.28 (s, 3H, retinoid 13-CH<sub>3</sub>), 3.15 (br s, 4H, -OH), 3.4-3.7 (m, 6H, pyranose H's), 4.75 (br t, 1H, J=9Hz, pyranose anomeric H), 5.85 (s, 1H, retinoid H-13), 6.04-6.33 (m, 4H, retinoid vinyl H's), 6.99 (dd, 1H, J=11.4, 15 Hz, retinoid H-11), 8.46 (br d), 1H, J=9 Hz, NH); RP-HPLC (CH<sub>3</sub>OH/H<sub>2</sub>O 9:1, both with 10 mM NH<sub>4</sub>OAc, flow rate 1 mL/min) tR = 6.4 min (96.4% of total peak area); FAB-MS, m/e (relative intensity) 484 (M + Na, 53.04).

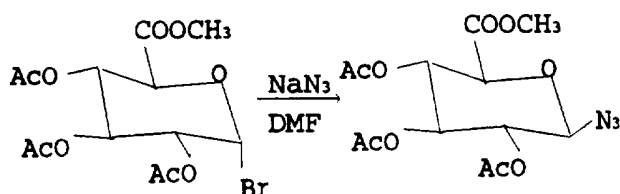
Example 8: 1-(D-glucopyranosyluronosyl) retinamide:

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First methyl-1,2,3,4-tetra-0-acetylglucuronate was prepared from glucurono-6,3-lactone by dissolving 40 gm of glucurono-6,3-lactone, available from Aldrich Chemical Co., in 300 mL of CH<sub>3</sub>OH containing 100 mg NaOH and allowed to stand one hour. The solvent was removed under reduced pressure and the residue dissolved in 100 mL pyridine and 150 mL acetic anhydride; the mixture was stored in the refrigerator. The resulting solid methyl-1,2,3,4-tetra-0-acetylglucuronate was filtered and recrystallized from 95% ethanol. The methyl-1,2,3,4-tetra-0-acetylglucuronate has a melting point of 178°C.

Methyl 1-bromo-2,3,4-tetra-0-acetylglucuronate was prepared from methyl-1,2,3,4-tetra-0-acetylglucuronate. 50 g methyl-1,2,3,4-tetra-0-acetylglucuronate was dissolved in 200 mL of 30% HBr/acetic acid; the mixture was allowed to stand overnight in the refrigerator. The solvent was removed under reduced pressure, the residue was dissolved in CHCl<sub>3</sub>, and the CHCl<sub>3</sub> solution was washed with H<sub>2</sub>O, saturated NaHCO<sub>3</sub>, saturated NaCl and dried over Na<sub>2</sub>SO<sub>4</sub>. The drying agent was removed by filtration and the residue crystallized from ethanol. The methyl 1-bromo-2,3,4-tetra-0-acetylglucuronate has a melting point of 107°C.

Preparation of methyl 2,3,4-tri-0-acetylglucopyranuronosyl azide



20

methyl 2,3,4-tri-0-acetylglucopyranuronosyl bromide

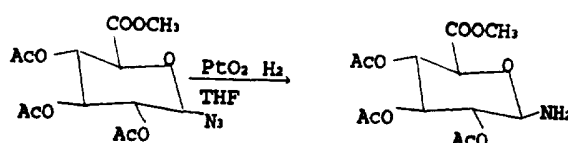
methyl 2,3,4-tri-0-acetylglucopyranuronosyl azide

Methyl 2,3,4-tri-0-acetylglucopyranuronosyl bromide (7.94 g, 20 mmol) was dissolved in 250 ml of dimethylformamide and 2.6 g, that is 40 mmol sodium azide was added. The mixture was stirred at room temperature for 24 hours and then poured into 500 ml of H<sub>2</sub>O. The organic soluble material was extracted three times with 150 ml ethyl acetate. The combined ethyl acetate extracts were washed twice with 100 ml H<sub>2</sub>O, brine, dried over MgSO<sub>4</sub>, and concentrated to dryness. The residue was recrystallized from ethanol to yield 5.46 g (76%) of methyl

2,3,4-tri-0-acetylglucopyranuronosyl azide. Methyl 2,3,4-tri-0-acetylglucopyranuronosyl azide had the following characteristics: melting point of 152-154°C; IR (KBr), 2972, 2958, 2123, 1761, 1740, 1375, 1244, 1213, 1070, 1039; <sup>1</sup>H NMR: δ2.0 (s, 6H), 2.06 (s, 3H), 3.76 (s, 3H), 4.10 (d, 1H, J=9.5 Hz), 4.69 (d, 1H, J=8.7 Hz), 4.94 (t, 1H, J=8.7 Hz), 5.23 (m, 2H).

Preparation of methyl 2,3,4-tri-0-acetylglucopyranuronosyl amine

Next, 6.17 g, that is, 17 mmol methyl 2,3,4-tri-0-acetylglucopyranuronosyl azide was added to 50 ml of dry tetrahydrofuran containing 400 mg of PtO<sub>2</sub>. The mixture was shaken under 40 psi of hydrogen for 2 hours, filtered, concentrated to dryness, to produce methyl 2,3,4-tri-0-acetylglucopyranuronosyl amine.



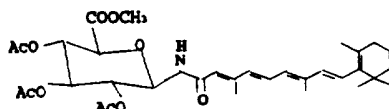
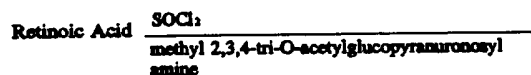
15 methyl 2,3,4-tri-0-acetylglucopyranuronosyl azide

methyl 2,3,4-tri-0-acetylglucopyranuronosyl amine

Preparation of methyl 2,3,4-tri-0-acetylglucopyranuronosyl retinamide

Retinoyl chloride was prepared by reacting 4.5 g, 15 mmol retinoic acid, 2 ml of pyridine, and 1.09 ml, that is, 15 mmol of thionyl chloride in 85 ml of ether at 0°C. The mixture was cooled to -15°C and 5.66 g, that is, 17 mmol methyl 2,3,4-tri-0-acetylglucopyranuronosyl amine was added in 70 ml of benzene containing 3.3 ml of pyridine. The mixture was stirred at room temperature for 72 hours, diluted with 300 ml of ethyl acetate, and extracted three times with 150 ml H<sub>2</sub>O and brine. The organic product was dried over MgSO<sub>4</sub>, concentrated, treated with diazomethane, and chromatographed with 40% ethyl acetate-hexane to yield 5.95 g or 65% of methyl 2,3,4-tri-0-acetylglucopyranuronosyl retinamide.

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**methyl 2,3,4-tri-O-acetylglucopyranuronosyl retinamide**

Preparation of 1-(D-glucopyranosyluronosyl) retinamide

5            Then    2.46    g,    4    mmol    of    methyl    2,3,4-tri-O-acetylglucopyranuronosyl retinamide was dissolved in 50 ml of methanol and 0.2 g of  $\text{K}_2\text{CO}_3$  was added. The mixture was stirred at room temperature for 6 hours and concentrated. The residue was dissolved in 50 ml of methanol. To this mixture was added 0.5 g, that is 9 mmol, of

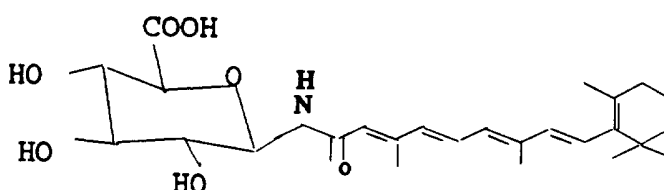
10    KOH and 50 ml of  $\text{H}_2\text{O}$  and the mixture was stirred for 24 hours. The mixture was acidified to pH 2 with 5% HCl and extracted three times with 100 ml ethyl acetate. The ethyl acetate extracts were dried over  $\text{MgSO}_4$  and concentrated to yield 1.58 g of 1-(D-glucopyranosyluronosyl) retinamide, (83%). The 1-(D-glucopyranosyluronosyl) retinamide was

15    purified by chromatography on RP-18 with 80% MeOH/ $\text{H}_2\text{O}$ . The 1-(D-glucopyranosyluronosyl) retinamide so obtained, is a mixture of  $\beta$  and  $\alpha$  epimers in approximately a 4 to 1 ratio.

1-(D-glucopyranosyluronosyl) retinamide has the following characteristics: melting point of  $145^\circ$  (dec); IR (KBr) 3390, 2925, 1733, 1652  $\text{cm}^{-1}$ ; UV ( $\text{CH}_3\text{OH}$ )  $\lambda_{\text{max}}$  351 nm ( $\epsilon$  38523);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  1.00 (s, 6H), 1.43 (m, 2H), 1.56 (m, 2H), 1.67 (s, 3H), 1.96 (br s, 5H), 2.28 (s, 3H), 3.21-3.76 (m, 8H), 4.78 (br t, 1H,  $J=8.9$  Hz), 5.83 (s, 1H), 6.07-6.33 (m, 4H), 6.95 (dd, 1H,  $J=11.7, 14.7$  Hz), 8.32 (br d, 0.2H,  $J=8.7$  Hz,  $\alpha$ -NH), 8.53 (br d, 0.8 H,  $J=9$  Hz,  $\beta$ -NH); RP-HPLC

20    ( $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  9:1, both with 10 mM  $\text{NH}_4\text{OAc}$ , flow rate 1 mL/min)  $t_r = 4.0$  min (96.1% of total peak area); FAB-MS,  $m/e$  (relative intensity) 552 (M-1 + 2K, 100).

1-(D-glucopyranosyluronosyl) retinamide



#### EVALUATION OF THE DRUGS

##### Stability of the Drugs

To determine the stability of the C-glucopyranosiduronate linkage towards acidic conditions, samples of 4-HPR-O-glucuronide, 4-(retinamido)phenyl-C-glucuronide and 4-(retinamido)benzyl-C-glucuronide were treated with 0.1 N methanolic HCl at 37°C for 2 hours.

After this time, the O-glucuronide had undergone substantial hydrolysis of the ether linkage to liberate the N-4-hydroxyphenylretinamide and glucuronic acid. However, both the 4-(retinamido)phenyl-C-glucuronide and the 4-(retinamido)benzyl-C-glucuronide remained essentially intact. Thus, both the C-phenyl and C-benzyl glycosides, which lack the ether linkage, resist acid hydrolysis and would withstand the low pH of the mammalian stomach.

Similarly, 1-(D-glucopyranosyluronosyl) retinamide and RAG were treated with 0.1 N HCl at 37°C for two hours.

After two hours, the RAG had undergone approximately 50% solvolysis to liberate retinoic acid as determined by HPLC. In contrast, the 1-(D-glucopyranosyluronosyl) retinamide remained intact.

While the 4-HPR-O-glucuronide is hydrolysed to glucuronic acid and the aglycone by  $\beta$ -glucuronidase, the 4-(retinamido)phenyl-C-glucuronide and the 4-(retinamido)benzyl-C-glucuronide are not. This indicates that the 4-(retinamido)phenyl-C-glucuronide and the 4-

-20-

(retinamido)benzyl-C-glucuronide would not be hydrolyzed by the enzyme in vivo.

Indeed, the 4-(retinamido)phenyl-C-glucuronide and 4-(retinamido)benzyl-C-glucuronide act as inhibitors of  $\beta$ -glucuronidase mediated hydrolysis of N-4-hydroxyphenylretinamide-O-glucuronide to N-4-hydroxyphenylretinamide and glucuronic acid with 4-(retinamido)benzyl-C-glucuronide being slightly more effective than the 4-(retinamido)phenyl-C-glucuronide. The  $K_m$  value of  $\beta$ -glucuronidase for 4-HPR-O-glucuronide under experimental conditions was determined to be 184.5  $\mu\text{M}$ , using the method of Beinfeld, P. et al., *J. Am. Chem. Soc.*, (1954), 76, 4872-4877. Accordingly, the concentration of the 4-HPR-O-glucuronide in the inhibition study was selected to be below 184.5  $\mu\text{M}$ . The  $\text{IC}_{50}$  values were determined according to the method of Lang, J.H. et. al. *J. Med. Chem.* (1971), 14, 223-236, using 10% methanol as a co-solvent, for 4-(retinamido)phenyl-C-glucuronide and 4-(retinamido)benzyl-C-glucuronide. The  $\text{IC}_{50}$  values, which represent the concentration of the drug that inhibits the cleavage of 50% of the 4-HPR-O-glucuronide, were found to be 267  $\mu\text{M}$  for the 4-(retinamido)phenyl-C-glucuronide and 236  $\mu\text{M}$  for the 4-(retinamido)benzyl-C-glucuronide. An average typical  $\text{IC}_{50}$  for glucaro-(1,4)-lactone, one of the most potent inhibitors of the enzyme, is 10  $\mu\text{M}$ . Thus, the  $\text{IC}_{50}$  values for the C-phenyl and C-benzyl glycosides, indicate that they significantly inhibit  $\beta$ -glucuronidase.

Similarly 1-(D-glucopyranosyluronosyl) retinamide and RAG were evaluated for their stability toward  $\beta$ -glucuronidase mediated hydrolysis under the same conditions as described above.

Within twenty minutes, the RAG was completely hydrolyzed to liberate retinoic acid, while the 1-(D-glucopyranosyluronosyl) retinamide remained intact.

### 30 Growth Inhibition of MCF-7 Cells

The antiproliferative activity of the drugs in MCF-7 human mammary tumor cell culture models were determined.

The MCF-7 cells were obtained originally from the Michigan Cancer Foundation. The cells were routinely grown as monolayers in T-25 plastic tissue culture flasks from Falcon, in Oxnard, CA containing 5.0 mL of B-10 medium consisting of Minimum Essential Medium (MEM, Earle's Base) supplemented with 1.0 mM MEM non-essential amino acid, 2.0 mM l-glutamine, 10% fetal bovine serum (FBS), and antibiotics. Culture conditions included a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C. One-half of the medium was replenished twice a week. Cells were harvested by

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trypsinization upon confluency. All variables were evaluated in triplicate.

For the growth inhibition assay the MCF-7 cells were plated in 24-well plates at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup>. After a 24 hour period and again on day 4, the medium was replaced with treatment medium containing the specified drugs at  $10^{-5}$  to  $10^{-8}$  M in absolute ethanol and added to the cell cultures so that the ethanol concentration did not exceed 0.1%. Control cultures were treated with an equivalent volume of absolute ethanol. On day 7 the cells were harvested by trypsinization and viability was assessed by trypan blue exclusion according to Ueda, H. et al. Cancer 1980, 46, 2203-2209. Total cell counts were obtained using a hemocytometer as well as a the Coulter Counter. Growth inhibition, that is, cell survival, was calculated for the various drug concentrations using the equation: % inhibition =  $100 - R/C \times 100$  where R and C are the number of cells in the drug-treated and control cultures, respectively.

The ED<sub>50</sub> for growth inhibition of the MCF-7 cells by the drugs has been evaluated in three separate assays as shown in Table I.

TABLE I  
GROWTH INHIBITION OF MCF-7 CELLS

DRUG	ED <sub>50</sub>
ASSAY 1	
4-HPR (control)	2.8 μM
4-(retinamido)benzyl-C-glucuronide	6.5 μM
4-(retinamido)benzyl-C-glucoside	3.4 μM
ASSAY 2	
4-HPR (control)	1.5 μM
4-(retinamido)benzyl-C-xyloside	15 μM
4-(retinamido)phenyl-C-glucoside	27 μM
ASSAY 3	
4-HPR (control)	33 μM
4-(retinamido)phenyl-C-glucuronide	50 μM

The 4-(retinamido)phenyl-C-glucuronide appears to show a potency consistent with its glucoside analogue, retinamidophenyl-C-glucoside. The 4-(retinamido)benzyl-C-glucuronide and its glucoside analogue, 4-(retinamido)benzyl-C-glucose exhibit the best growth inhibition of the cells. The glucuronides and glycosides drugs of the present invention are more effective than the xylosides of the present invention and the benzyl drugs are more active than the phenyl drugs.

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Although the mechanism of the drugs is not well understood, it has been shown that active drugs in this class compete with retinoic acid for nuclear receptors for retinoic acid.

Retinoic acid receptors, (RAR)  $\alpha$ ,  $\beta$ , and  $\gamma$  were expressed using a baculovirus expression system in *Spodoter frugiperda* insect cells. DNA, coding for each receptor was introduced into the genome of the baculovirus which was then introduced into the cultured insect cells. Whole cell extracts were prepared by homogenizing cells in 50 mM Tris buffer at pH 7.4 containing 1.5 mM EDTA, 5 mM dithiothreitol, 300 mM KCl and the protease inhibitors, 1 mM phenylmethylsulfonyl fluoride and 5 ug/ml soybean trypsin inhibitor. The homogenate was subjected to centrifugation at 45,000 rpm for 45 minutes at 4°C in a Ti 70.1 Beckman rotor. The supernatant fraction was stored at -70°C until use. For drug competition binding studies, 500  $\mu$ l portions of the retinoic acid receptor-containing protein extracts were incubated on ice for 3 hours in the presence of 5 nM [<sup>3</sup>H] trans-retinoic acid, from DuPont-NEN, 50.6 Ci/mmole) (RA)  $\pm$  1  $\mu$ M of each of the drugs listed in Table II or the equivalent volume of ethanol. Ethanol comprised 3.8% of the final volume in each tube. The [<sup>3</sup>H]-RA-receptor complexes were separated from free ligand using an hydroxylapatite assay, (Williams, D. et al., *Biochemistry* (1974) 13, 5537-5542, and Dame, et al., *Proc. Natl. Acad. Sci. USA* (1985) 82, 7825-7829). The quantity of [<sup>3</sup>H]-RA bound by protein was determined by liquid scintillation counting in 4 ml of Optifluor from Packard, Meridan, CT. The results are shown in Table II.

TABLE II  
DRUG COMPETITION WITH (<sup>3</sup>H)RETINOIC ACID  
EXPRESSED AS PERCENT REDUCTION OF BINDING  
OF THE (<sup>3</sup>H)RETINOIC ACID

DRUG* 1 $\mu$ M	mRAR $\alpha$	mRAR $\beta$	mRAR $\gamma$
retinoic acid	100%	100%	100%
4-HPR	18	5	7
4-HPR-O-glucuronide	14	4	7
4-(retinamido)phenyl-C-glucuronide	48	47	40
4-(retinamido)benzyl-C-glucuronide	67	82	78
4-(retinamido)benzyl-C-glucoside	56	64	77
4-(retinamido)benzyl-C-xyloside	12	3	2
13-cis-retinoic acid	28	13	4

If the drugs of this invention exert their chemopreventative effect by binding to receptors for retinoic acid, then the data supports the preference for 4-(retinamido)phenyl-C-glucuronide, 4-

(retinamido)benzyl-C-glucoside and particularly 4-(retinamido)benzyl-C-glucuronide, which significantly compete with retinoic acid for the receptors.

5 1-(B-D-glucopyranosyl) retinamide, and 1-(D-glucopyranosyluronosyl) retinamide were tested for their ability to bind to the nuclear retinoic acid receptors  $\alpha$ ,  $\beta$  and  $\gamma$ . The human retinoic acid receptor cDNA was obtained by reverse transcription/PCR amplification using RNA from MCF-7 cells. The coding region of the cDNA was subcloned into the baculovirus vector pVL1393. Production of  
10 the receptor protein, human retinoic acid receptor $\gamma$ /BCV, followed conventional procedures. Human retinoic acid receptor $\gamma$ /BCV exhibits the expected characteristics of a retinoic acid receptor, that is, it: had a molecular size of -50 kD by SDS-PAGE; demonstrated specific, high-affinity binding to retinoic acid ( $K_d = 0.76$  nM), and demonstrated  
15 specific DNA-binding as shown by gel mobility shift of labeled retinoic acid response element DNA. Generation of retinoic acid receptors  $\alpha$  and  $\beta$ , and competition binding methodology are described in Proc. Nat'l Acad. Sci. USA, 1993, 90, 7293.

20 Competition binding analyses were performed using 650 moles recombinant retinoic acid receptor proteins, for 1.5  $\mu$ g total protein incubated with 1.3 nM [ $^3$ H]-retinoic acid and increasing concentrations of unlabeled retinoic acid, 1-(B-D-glucopyranosyl) retinamide and 1-(D-glucopyranosyluronosyl) retinamide. The results were graphed and are presented in Figure 1.

25 As shown in Figure 1, 1-(B-D-glucopyranosyl) retinamide, and 1-(D-glucopyranosyluronosyl) retinamide were 1500- and -500-fold less effective than retinoic acid in competing for [ $^3$ H]-retinoic acid binding to retinoic acid receptor  $\gamma$ . The  $K_i$  constant was 0.5 nM for retinoic acid, 710 nM for 1-(B-D-glucopyranosyl) retinamide, and 280 nM for 1-  
30 (D-glucopyranosyluronosyl) retinamide.

As shown in Figures 2 and 3, similar results were observed for binding of 1-(B-D-glucopyranosyl) retinamide, and 1-(D-glucopyranosyluronosyl) retinamide by retinoic acid receptors  $\alpha$  and  $\beta$ .

35 The drugs of the present invention while described as cancer preventatives and cancer treatments, may also be used in the study of cancer, particularly breast cancer.

#### Evaluation in-vivo

40 To determine the effect of 1-(B-D-glucopyranosyl) retinamide, 1-(D-glucopyranosyluronosyl) retinamide and retinoic acid on the course of mitogen induced mammary tumor development, female rats were intubed with a single 15 mg oral dose of 7,12-dimethylbenz(a)anthracene (DMBA). The rats were then fed 1 mmol/Kg of either 1-(B-D-glucopyranosyl) retinamide, or 1-(D-glucopyranosyluronosyl) retinamide, and retinoic acid in the diet from 10 days before through 110 days after intubation.

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The control rats received DMBA intubation only and AIN-76A rat chow. The results are summarized in Table III.

Table III. Effects of Retinoids on Progression of DMBA-Induced Rat Mammary Tumors.

	compound	tumor latency (days)	tumor incidents (%)	tumor number/rat
5	control	42	92	1.50
	retinoic acid	49	83	1.17
	1-(B-D-glucofuranosyl) retinamide	64	58	0.92
10	1-(D-glucofuranosyluronic acid) retinamide	64	50	0.83

The administration of 1-(B-D-glucofuranosyl) retinamide, and 1-(D-glucofuranosyluronic acid) retinamide significantly prolonged the latency period of mammary cancer. In control rats, the first tumors appeared at 42 days post intubation, and 92% of the rats had tumors by 110 days post intubation. In rats that were treated with 1-(B-D-glucofuranosyl) retinamide, and 1-(D-glucofuranosyluronic acid) retinamide, tumor induction was markedly delayed and reduced as opposed to both the control rats and the rats that received retinoic acid. The first tumors were not observed until 64 days post DMBA intubation in the rats that received 1-(B-D-glucofuranosyl) retinamide, and 1-(D-glucofuranosyluronic acid) retinamide. 58% of the rats that received 1-(B-D-glucofuranosyl) retinamide and 50% of the rats that received 1-(D-glucofuranosyluronic acid) retinamide had developed tumors by 110 days post intubation. 1-(D-glucofuranosyluronic acid) retinamide caused the largest reduction in multiple mammary tumors. At 110 days post DMBA intubation, the control rats had an average of 1.5 tumors per rat compared to 0.83 tumors per rat in animals receiving 1-(D-glucofuranosyluronic acid) retinamide, to 0.92 tumors per rat in animals receiving 1-(B-D-glucofuranosyl) retinamide. 1-(B-D-glucofuranosyl) retinamide, and 1-(D-glucofuranosyluronic acid) retinamide show a greater mammary tumor chemopreventive effect than does retinoic acid. 1-(B-D-glucofuranosyl) retinamide, and 1-(D-glucofuranosyluronic acid) retinamide inhibit DMBA induced mammary tumor development.

Although certain embodiments of this invention have been shown and described, various adaptations and modifications can be made without departing from the scope of the invention as defined in the appended claims.

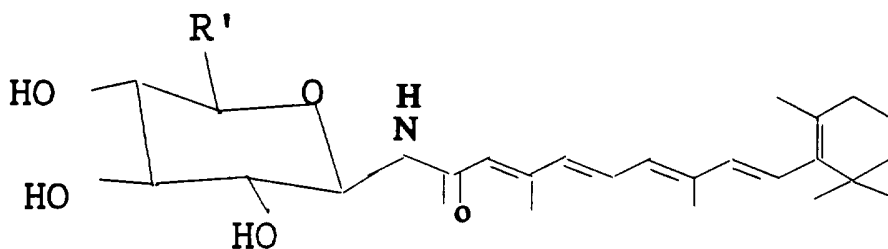
Furthermore, other isomers of the structures disclosed herein are within the scope of this application and include, but are not limited to: 1-( $\beta$ -D-mannopyranosyl) retinamide; 1-( $\beta$ -D-mannopyranuronic acid)

retinamide; 1-( $\beta$ -D-galactopyranosyl) retinamide; 1-( $\beta$ -D-galactopyranuronosyl) retinamide; and 1-( $\beta$ -D-xylopyranosyl) retinamide.

CLAIMS

What is claimed is:

1. A composition of matter having the following structure:

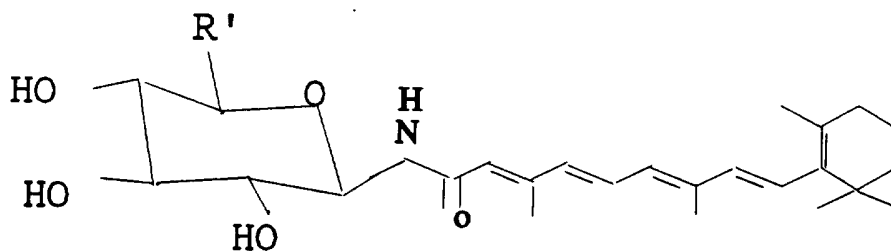


wherein: R' is H, COOH, or CH<sub>2</sub>OH;  
or isomers thereof.

2. The invention of Claim 1, wherein R' is an CH<sub>2</sub>OH group.
3. The invention of Claim 1, wherein R' is a COOH group.
4. A method for preparing a composition of matter comprising the steps of:

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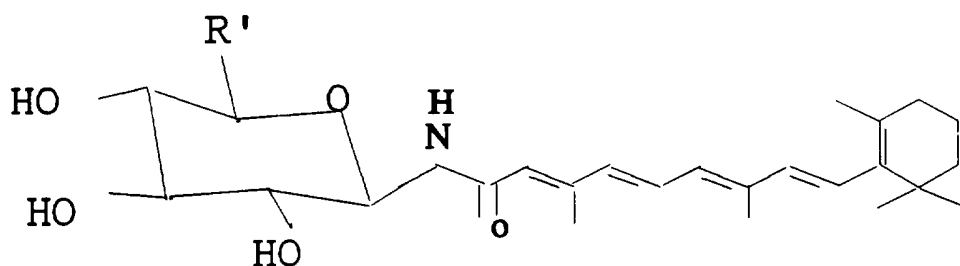
- A. providing a glycoside wherein all the hydroxy groups are protected;
  - B. placing an azide group on the protected glycoside;
  - C. reducing the product of step B to its corresponding amine;
  - D. retinoylating the product of step C; and
  - E. deprotecting the product of step D.
5. The invention of Claim 4, wherein the composition of matter has the following structure:



wherein R' is H, COOH, or CH<sub>2</sub>OH, or isomers thereof; wherein the glycoside is peracetylated, and the deprotection of step E is accomplished by deacetylating the product of step D.

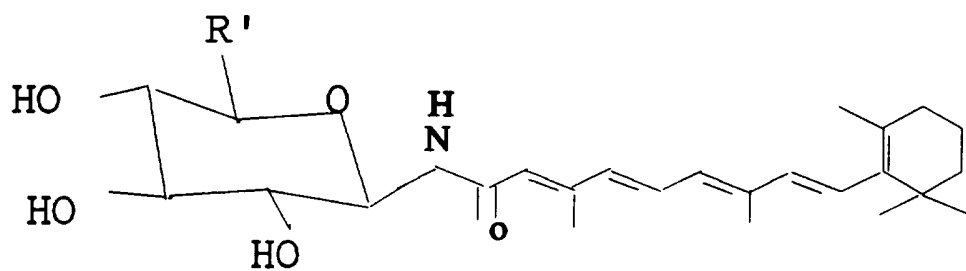
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6. The invention of Claim 5, wherein R' is an CH<sub>2</sub>OH group.
7. The invention of Claim 5, wherein R' is a COOH group.
8. A method for treating or preventing breast cancer in patients comprising the steps of:
  - a. providing a drug having the following structure:



wherein: R' is H, COOH, or CH<sub>2</sub>OH; or isomers thereof;

- b. administering the drug to the patient.
9. A drug for the prevention or treatment of mammalian breast cancer having the following structure:



wherein: R' is H, COOH, or CH<sub>2</sub>OH; or isomers thereof.

FIG. 1

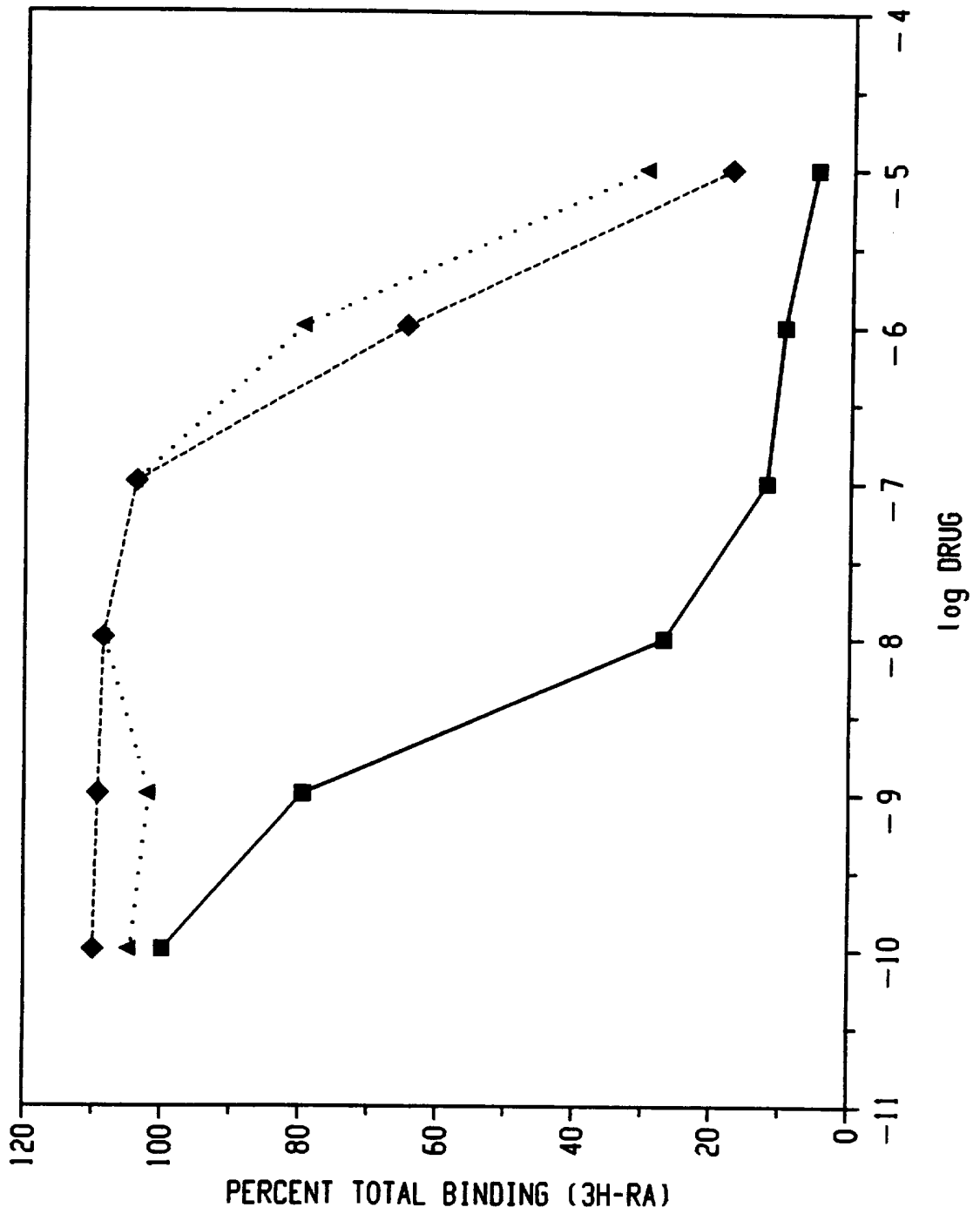


FIG. 2

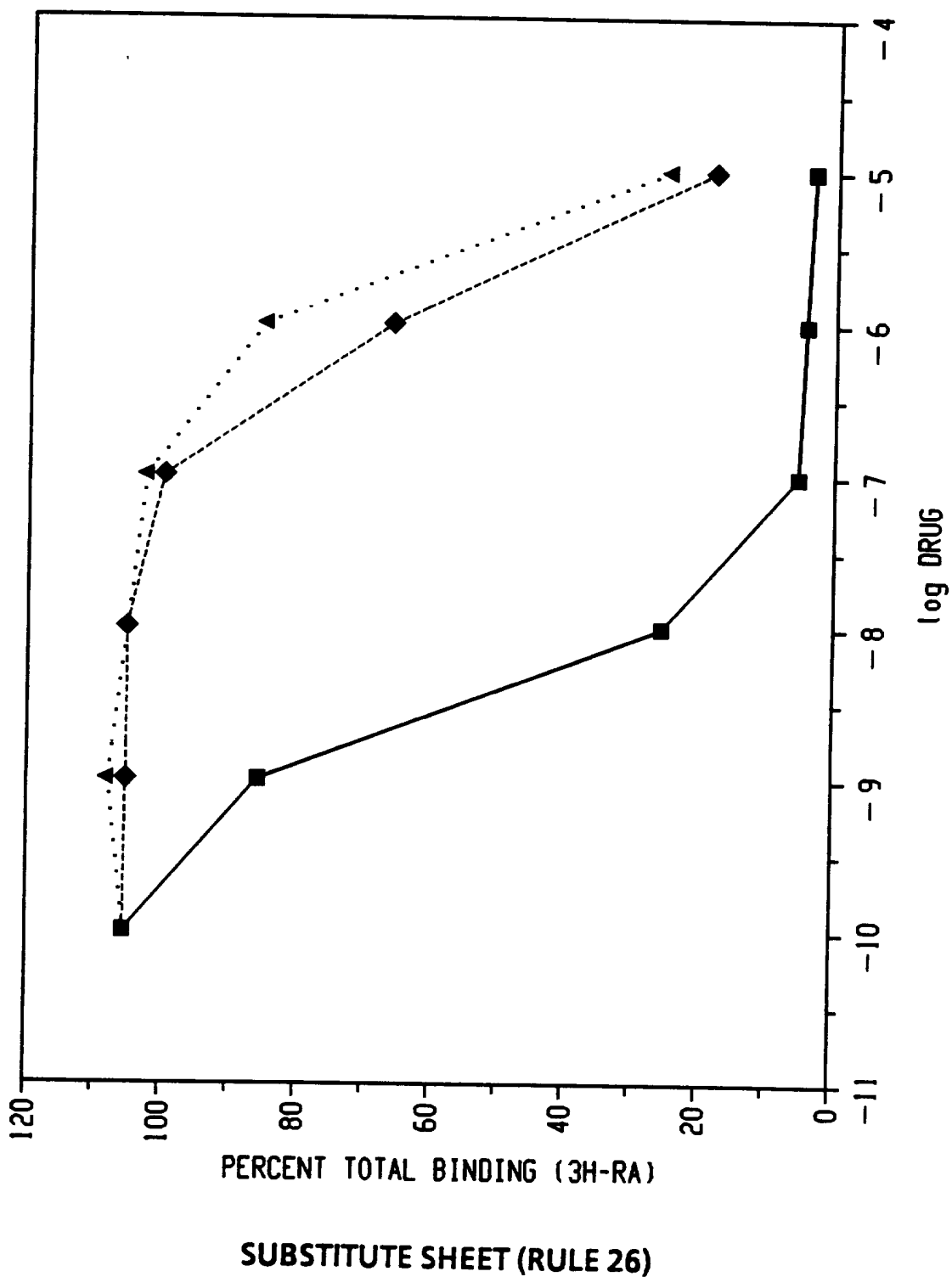
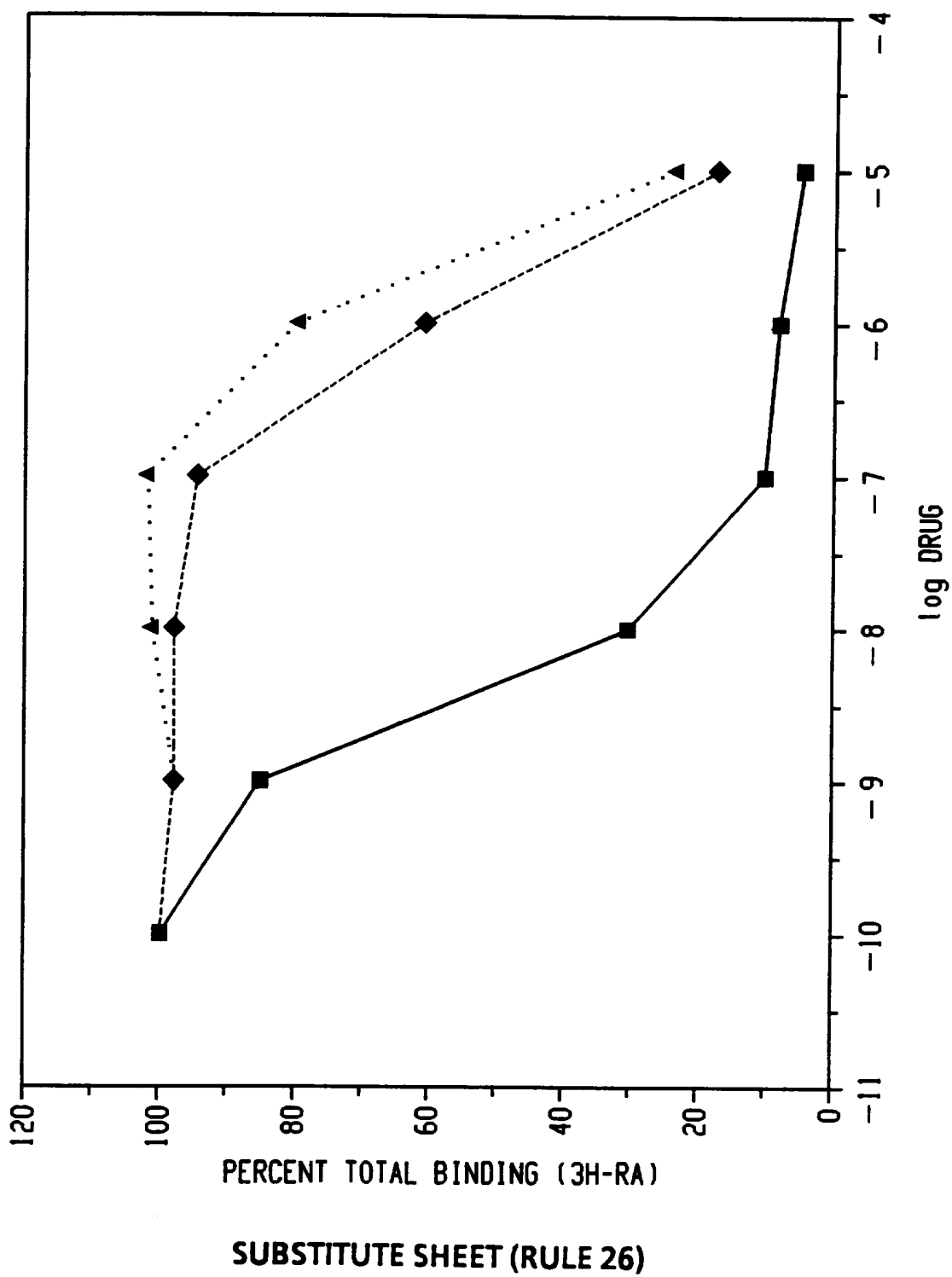


FIG. 3



INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/12258

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) .A61K 31/35; C07D 309/14.  
US CL :514/459; 549/417.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/459; 549/417.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE: THE STRUCTURAL FORMULA.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF LIPID RESEARCH , Volume 26 , issued 1985, ARUN B. BARUA ET AL, "Chemical Synthesis of all-trans retinoyl Beta-glucuronide", pages 1277-1282.	1-7

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 DECEMBER 1995

Date of mailing of the international search report

03 JAN 1996

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