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IDENTIFICATION OF AN ACTIVATOR OF THE RETINOID X RECEPTOR

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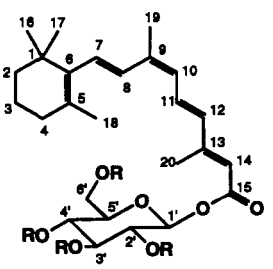
ABSTRACT.—A metabolite of all-*trans* retinoic acid produced by insect cells was discovered to activate the intracellular retinoic acid X receptor. The compound, β-glucopyranosyl 9-*cis*-retinoate [**1**], was purified and its structure was determined by analysis of spectroscopic data.

Intracellular receptors (IRs) are a superfamily of transcription factors that are responsible for regulating a variety of complex physiological functions (1,2). IRs initiate the modulation of gene transcription upon binding of their respective ligands, which include small molecules such as steroids, retinoic acid, and vitamin D. Retinoic acid receptors have been discovered recently as members of the superfamily of IRs (3,4). Two subfamilies of retinoic acid receptors, RAR and RXR, have been identified, with each subfamily containing three isoforms designated α,β, and γ. All-*trans*-retinoic acid (ATRA) activates gene transcription by binding to members of the RAR subfamily but does not bind to RXRs (5–9). However, at high concentrations, ATRA activates transcription of the RXRs. Thus, it was postulated that a metabolite of ATRA, generated *in situ,* directly binds to RXR resulting in modulation of gene expression. This led to our recent identification of 9-*cis*-retinoic acid (9-*cis*-RA) as a high-affinity ligand for the retinoid X receptor, RXR (8). Herein, we describe a continuation of the study of retinoic acid metabolism by insect cells that has led to the identification of β-glucopyranosyl 9-*cis*-retinoate [**1**] as a potent activator of RXR.



1 R=H
2 R=Ac

During the previous study (8), it was discovered that an organic extract of *Drosophila melanogaster* Schneider cells incubated with ATRA contained a RXR-active metabolite that was chromatographically very different from 9-*cis*-RA. It was also discovered that when the cells were incubated with 9-*cis*-RA instead of ATRA, the abundance of this metabolite increased as indicated by hplc analysis of the crude organic extract. During our search for a cell type that would produce a higher yield of this metabolite, SF21 cells, another type of insect cells, were found to produce ten times more metabolite than Schneider cells. Therefore, in order to facilitate the isolation of this metabolite, later incubations were performed in SF21 cells with 9-*cis*-RA.

The EtOAc extract of the cell pellet obtained after an incubation period of 24 h was separated by hplc on reversed-phase Si gel (C-18) using MeCN-MeOH-2% AcOH in H_2O (56:16:28). The ester [**1**] was obtained as a yellow amorphous solid after freeze-drying the hplc fraction. The molecular formula of **1**, $C_{26}H_{38}O_7$, was determined by fabms, which showed a $[M+H]^+$ ion at *m/z* 463.2657 (calcd 463.2697). The ir spectrum of **1** (film) indicated the presence of OH (3360 cm^{-1}, br) and C=O (1721 cm^{-1}). The presence of a 9-*cis*-RA moiety in **1** could be easily identified by compar-

ing the ^{1}H-nmr spectra of the two compounds (10). However, **1** did not respond to methylation with CH_2N_2, indicating the absence of a free carboxyl group. The resonances in the ^{1}H-nmr spectrum that belonged to the rest of the molecule were between δ 3 and 5.5 ppm (see Table 1), which indicated that the second fragment of the molecule was highly oxygenated. The correlations from a ^{1}H-nmr COSY experiment were used in the identification of this fragment as a hexose moiety. Analysis of the coupling constants of the ^{1}H-nmr resonances assigned to the hexose fragment revealed glucose as the hexose (11), and hence **1** must be an ester of 9-*cis*-RA (see Table 1).

The resonances assigned to the C-6′ methylene protons of glucose sharpened upon addition of D_2O into the nmr sample in $CDCl_3$, indicating the presence of a free OH group at C-6′. Upon acetylation with Ac_2O in pyridine, **1** yielded the tetraacetate **2**. In the ^{1}H-nmr spectrum of **2**, significant acetylation shifts were observed for protons at C-2′, C-3′, and C-4′, but only a 0.4 ppm acetylation shift was observed for H-1′ (see Table 1). This would be expected only if the attachment of the ester functionality to the sugar were at C-1′ (12).

Figure 1 shows the dose response curves obtained for 9-*cis*-RA and **1** on RXRα in a co-transfection assay. Both compounds activated the two receptors, RAR and RXR, with similar potencies for each receptor. The observation of similar behavior for 9-*cis*-RA and **1** appears to indicate that the sugar moiety in **1** plays only a very minor role, if any, in the transcriptional activation of RAR and RXR. We were intrigued by the ability of SF21 cells to produce **1** with high efficiency, and we believe the conversion

TABLE 1. ^{1}H-Nmr Data[a] for the β-Glucopyranosyl 9-*cis*-retinoates **1** and **2**.

Position	**1**	**2**
	δ ppm	δ ppm
2	1.48 m (2H)	1.48 m (2H)
3	1.63 m (2H)	1.63 m (2H)
4	2.05 m (2H)	2.08 m (2H)
7	6.80 d, 16 (1H)	6.80 d, 16 (1H)
8	6.35 d, 16 (1H)	6.36 d, 16 (1H)
10	6.15 d, 12 (1H)	6.15 d, 12 (1H)
11	7.29 dd, 12, 15 (1H)	7.32 dd, 12, 15 (1H)
12	6.39 d, 15 (1H)	6.39 d, 15 (1H)
14	5.82 s (1H)	5.78 s (1H)
16	1.03 s (3H)	1.03 s (3H)
17	1.03 s (3H)	1.03 s (3H)
18	1.74 br s (3H)	1.74 br s (3H)
19	2.02 br s (3H)	2.03 br s (3H)
20	2.34 br s (3H)	2.34 br s (3H)
1′	5.54 d, 8.5 (1H)	5.94 d, 8.5 (1H)
2′	3.35 dd, 8.5 (1H)	5.08 m (1H)
3′	3.48 dd, 8.5 (1H)	5.39 dd, 9 (1H)
4′	3.40 dd, 8.5 (1H)	5.08 m (1H)
5′	3.39 m (1H)	4.13 m (1H)
6′	3.66 dd, 12, 1 (1H)	4.09 dd, 12, 5 (1H)
	3.79 dd, 12, 3.5 (1H)	4.26 dd, 12, 1.5 (1H)
CH_3(Ac)		1.95 s (3H)
		1.97 s (3H)
		2.00 s (6H)

[a]Chemical shifts are reported with reference to internal TMS at δ 0.0 in $CDCl_3$, at 500 MHz. Coupling constants are given in Hz after multiplicities.

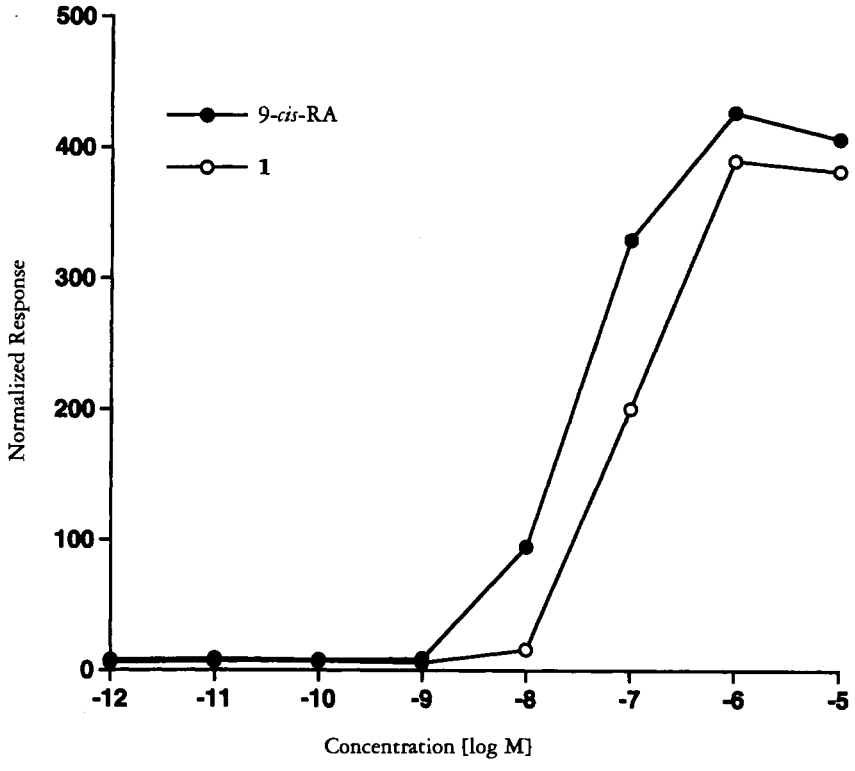


FIGURE 1. Transcriptional activation of RXRα by 9-*cis*-RA and **1** in an in vitro co-transfection assay in mammalian cells.

of 9-*cis*-RA to **1** might provide a means of storage of 9-*cis*-RA in these cells. To the best of our knowledge, **1** is unprecedented in the literature.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-Nmr experiments were performed on a Varian Unity 500 MHz instrument. A Perkin-Elmer 1600 series Ft-ir instrument was used to acquire ir spectra. All the solvents used in the extractions and chromatography were Mallinckrodt hplc grade solvents. Hplc was performed on reversed-phase ODS columns (5μm, 4.6 mm i.d.×25 cm), and the peaks were monitored using absorbance at 350 nm.

FERMENTATIONS.—Thirteen spinner flasks each containing 500-ml cultures of SF21 insect cells at a density of 1.5 to 2 million cells/ml were prepared and 500 μl from a 5mM stock solution of 9-*cis*-RA were added to each flask. Cells were harvested after a 24-h incubation period at 37°.

EXTRACTION AND ISOLATION.—The cells were removed from the fermentation broth by centrifugation. The cell pellet was extracted with EtOAc (3×50 ml), and the combined EtOAc extracts were concentrated *in vacuo* to obtain the crude organic extract, which was re-immersed in MeOH for purification by hplc. The crude extract was separated by hplc using an ODS column and MeCN-MeOH-2% AcOH in H_2O (56:16:28) as the eluent at a flow rate of 1.2 ml/min to obtain 500 μg of pure **1**, which had a R_t of 15 min.

CO-TRANSFECTION ASSAY.—Assays were performed exactly as described in a previous study (8). Reporter-luciferase constructs used were the palindromic thyroid hormone response element (TRE-pal) for RAR cotransfections, and the CRBPII response element for RXR cotransfections.

2′,3′,4′,6′-Tetraacetoxy-β-glucopyranosyl 9-cis-*retinoate* [**2**].—The ester [**1**] (500 μg) was dissolved in 0.5 ml of Ac_2O and five drops of pyridine. The mixture was stirred at room temperature for about 12 h. The excess reagents were then removed by concentration *in vacuo* to yield 600 μg of **2** as an oil: ^{1}H nmr (see Table 1), fabms: *m/z* 653.4 (M^++Na).

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