Isolation, Structure, Synthesis, and Bioactivity of a Novel Putative Insulin Mediator. A Galactosamine chiro-Inositol Pseudo-Disaccharide Mn^{2+} Chelate with Insulin-like Activity[#]

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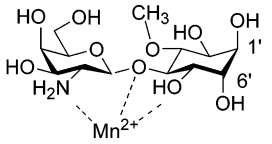
We isolated from beef liver a putative insulin mediator termed INS-2, **1**. Its structure was determined to be a novel inositol glycan pseudo-disaccharide Mn^{2+} chelate containing D-chiro-inositol **2a** (as pinitol) and galactosamine. Purification methods were scaled up from those previously reported to isolate an inositol glycan with similar composition from rat liver.[1] Structure of the beef liver glycan was determined by degradative chemistry and 2D NMR spectroscopy and confirmed by chemical synthesis. Its structure is 4-*O*-(2-amino-2-deoxy-*β*-D-galactopyranosyl)-3-*O*-methyl-D-chiro-inositol **1** (INS-2, Figure 1). Its role as an insulin mimetic was demonstrated by its action in vivo to decrease elevated blood glucose injected to low-dose streptozotocin diabetic rats in a stereospecific and dose-dependent manner. The pseudo-disaccharide also stimulated [^{14}C]glucose incorporation into [^{14}C]glycogen in a dose-dependent manner in H4IIE hepatoma cells in the presence of insulin, thus enhancing insulin action. Only when chelated to Mn^{2+} did it activate pyruvate dehydrogenase phosphatase in vitro in a dose-dependent manner. To our knowledge, this is the first example of a *β*-1,4-linked inositol glycan consisting of D-chiro-inositol and galactosamine isolated from animal tissues with insulin mimetic actions.

Introduction

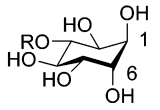
An intracellular insulin-mediated signaling system involving the intermediacy of molecules known as putative insulin mediators (PIMs) has been proposed to operate together with the tyrosine kinase cascade system.[2] By this mechanism of hormone action, insulin regulates the cleavage of a glycolipid or proteinated precursor to generate diacylglycerol and inositol glycan (IPG) putative second messengers. The precise chemical structures of these putative mediators have not previously been reported. However, two separate families of PIMs have been identified on the basis of two inositol and two hexosamine components. Type-A IPGs, so-called because of their ability to inhibit cyclic AMP (cAMP) dependent protein kinase, contain myo-Inositol and glucosamine.[3] Type-P IPGs contain D-chiro-inositol **2a** and galactosamine and activate pyruvate dehydrogenase (PDH) phosphatase.[4] The partial characterization of these putative mediators suggests structural similarities with glycosylphosphatidylinositols (GPIs).[5]

Type-A IPGs may arise from GPI anchors, which serve to attach proteins to the outer face of cell membranes through a covalent linkage between the protein and a phosphoethanolamine residue of the glycan component of the GPI. The sequential action of a protease and a phospholipase then liberates the IPG.[5] Because the glycan chain is highly conserved in all known examples of GPI anchors, involving the linear structural motif of Man-α(1,2)-Man-α(1,6)-Man-α (1,4)-$GlcNH_2$-α(1,6)-myo-Ino **3**, synthetic efforts have been directed at variations of compound **3**. This includes several syntheses of the polar headgroup pseudo-disaccharide.[6]

Previously, using separate enzyme assays, we purified from rat liver two inositol glycan species that were increased following in vivo insulin administration.[1] One, which contained myo-Inositol and glucosamine, inhib-



INS-2, 1



2a: D-chiro-inositol, R=H
2b: D-pinitol, R = CH_3

Figure 1. Structures of putative insulin mediator **1** (INS2), D-chiro-inositol **2a**, and D-pinitol **2b**.

[#] Dedicated to H. E. "Herb" Carter who introduced one of us (J.L.) to inositol chemistry and biochemistry.

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ited cAMP-kinase (type A).[3] The second, which contained D-chiro-inositol and galactosamine, stimulated PDH phosphatase, PP2C, and indirectly PP1 (type P).[4] Its in vitro action on PDH phosphatase mimicked the action of insulin on fat tissue segments.[4a,7] Both of these species were also active in vivo to decrease hyperglycemia in low-dose streptozotocin (STZ) diabetic rats.[8]

In the present work, we scaled up the isolation protocol and isolated from fed beef liver an inositol glycan with insulin-mimetic properties in vivo and in vitro. Its structure was determined as a Mn^{2+} chelate of 4-*O*-(2-amino-2-deoxy-*β*-D-galactopyranosyl)-3-*O*-methyl-D-chiro-inositol (INS-2) **1**, by chemical degradation, 2D NMR spectroscopy, and chemical synthesis. Although clearly active at the micromolar level as an insulin mimetic, we believe it is chemically related to an even more active inositol glycan species previously described from our laboratory.[8b] Here, we report it as a novel inositol glycan species with insulin mimetic properties. *It is the first example of a putative insulin mediator derived from insulin-sensitive tissue that has been completely characterized.* In a subsequent paper, we will detail studies on the docking of INS-2 into the crystal structure of phosphoprotein phosphatase 2C and propose an allosteric mechanism for activation of the enzyme.

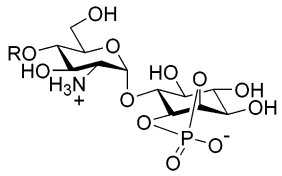
Results

Isolation of a D-chiro-Inositol Glycan Putative Insulin Mediator from Beef Liver. Fed (glycogen rich-"insulinized") beef liver was obtained from a slaughterhouse, cut into pieces, and immediately frozen in liquid nitrogen. Beef liver obtained from the supermarket was deemed unsuitable because, on analysis, it was shown to be essentially glycogen-free.

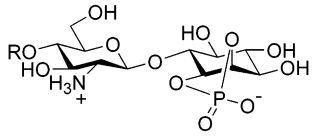
Small sections of frozen liver, 1 kg, were added to 3 L of solution A containing 50 mM formic acid, 1 mM mercaptoethanol, and 1 mM Na_2EDTA (pH 7.0) mixed in a blender for 2 min, transferred to a quart boiling pot, brought rapidly to a boil, and boiled for 5 min. After cooling, the mixture was centrifuged at low speed to remove denatured protein, treated with activated charcoal (10 mg/mL) at 4 °C for 10 min to remove nucleotides, and then centrifuged to remove the charcoal. The supernatant was adjusted to pH 6 and recentrifuged.

To the filtered supernatant, 10 L of deionized H_2O was added containing [$^{32}P_i$] (500 cpm/mL). Prewashed resin, AG1•formate form, was added (700 mL/kg original liver weight). The mixture was kept overnight at 4 °C with constant stirring. In the morning, an aliquot of the supernatant was analyzed for [$^{32}P_i$] to determine the extent of adsorption. Typically, 90−95% of the [$^{32}P_i$] was found to have been adsorbed. The resin was then poured onto a 65 mm × 400 mm chromatography column, and the flow-through was collected. Elution was carried out with five bed volumes of pH 2.0 HCl followed by five bed volumes of pH 1.3 HCl. Both HCl fractions were initially concentrated to small volumes on a rotary evaporator at 35−40 °C and then frozen and lyophilized to dryness. Both PDH phosphatase bioactivity and chiro-inositol content were determined on these and subsequent fractions.

To remove excess free metals, the pH 2.0 eluate was then subjected to chelex fractionation as follows. Chelex



3a: R = Man-α-(1,2)-Man-α-(1,2)-Man-α-(1,6)-Man-α-1-
3b: R = Man-α-(1,2)-Man-α-(1,6)-Man-α-1-



4: R = Man-α-(1,2)-Man-α-(1,2)-Man-α-(1,6)-Man-α-1-

Figure 2. Insulin mimetic glycosylphosphatidylinositol anchors.

100 (Biorad) was prewashed with 1 N HCl followed by water till it was neutral. The pH 2.0 eluate redissolved in H_2O was neutralized to pH 6.0 and then applied to a chelex column (1.5 cm × 22 cm). The column was eluted with 200 mL of 0.1 N HCl. The 0.1 N HCl eluate was collected in small fractions and lyophilized immediately. The pass-through and acid eluate were pooled and neutralized to pH 6.0. To increase recovery, the combined pass-through/eluate was applied to a second chelex column (1.5 cm × 22 cm). After washing with 200 mL of water, the column was eluted with 200 mL of 0.1 N HCl. Again, small fractions were collected and lyophilized. The lyophilized fractions were dissolved in water and bioassayed on PDH phosphatase. Fractions that stimulated PDH phosphatase were combined.

A silica column step was next used to remove contaminating peptides. The combined chelex eluates were reconstituted in a small volume of H_2O (pH 6), mixed with dry silica to make a slurry, and then vacuum-dried. The silica was transferred to the top of a prepacked column (22 mm × 170 mm) of dry silica and eluted with five bed volumes of solvent containing pyridine/2-propanol/acetic acid/water (8:8:1:4). Fractions (5−10 mL) were monitored by analytical TLC to detect the presence of the inositol glycan and to verify the removal of peptide material. The early fractions 1 and 2 (Figure 3, lanes 2 and 3) contained the bulk of the high R_f inositol glycan material (peach ninhydrin staining) with little of the low R_f peptide material (blue ninhydrin staining) as seen in fraction 3 (Figure 3, lane 4).

These early fractions were pooled, frozen, and lyophilized. The lyophilizate was reconstituted as above in water (pH 6), applied to a Biogel P4 sizing column (16 mm × 98 cm), and eluted with water (pH 6), collecting 2.5 mL fractions. Typically, the bioactive material stimulating PDH phosphatase and containing chiro-inositol was recovered in fractions 52−58. The active fractions were pooled, frozen, and lyophilized and then redissolved in a small volume of water (pH 6). HPLC chromatography was performed over a Waters Protein-Pak column with water (pH 6) elution. One milliter fractions were collected with the bioactive putative mediator present in fractions 17 and 18. There were large increases in bioactivity after the chelex and silica

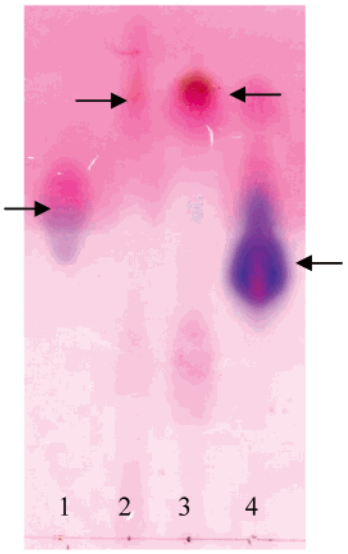
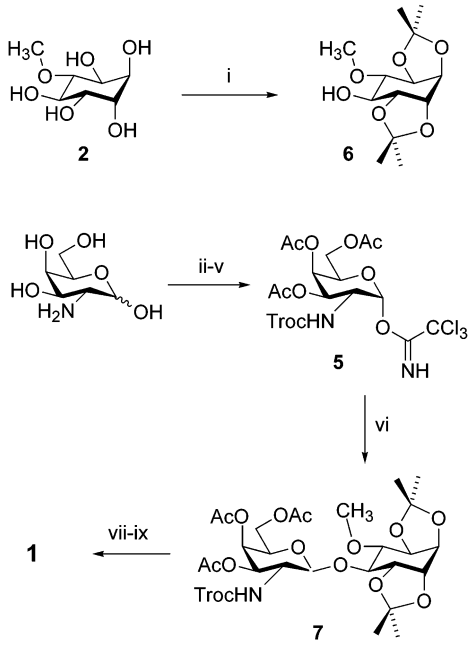


Figure 3. TLC analysis of silica column fractions from beef liver. Aliquots of fractions 1−3 that eluted from the silica column, stained with ninhydrin together with a galactosamine standard, are shown. TLC solvent used was pyr/2-propanol/HOAc/H_2O (8:8:1:4). Lane 1: galactosamine standard. Lanes 2−4: fractions 1−3 eluated from the silica column. Note the higher R_f peach staining spots in lanes 2 and 3, with the most prominent in lane 3 separated from the heavier blue staining peptide material in lane 4, fraction 3. See the text for further discussion.

Scheme 1[a]



[a] Reaction conditions: (i) DMP, acetone, pTsOH; (ii) TrocCl, $NaHCO_3$, H_2O; (iii) Ac_2O, pyr (1:1); (iv) H_2NNH_2·HOAc, DMF; (v) Cl_3CCN, DBU, CH_2Cl_2; (vi) **6**, 4 Å molecular sieves, TMSOTf, CH_2Cl_2; (vii) 80% aqueous AcOH; (viii) Amberlite 420 (OH form), 2-propanol/H_2O (1:5); (ix) pH 6.8, $MnCl_2$(aq).

column steps due to removal of inhibitory material. The total chiro-inositol content decreased throughout the purification. The specific activity increased significantly through the P4 column and HPLC steps, achieving a 70-fold increase over the starting material.

Chemical Composition Studies. Acid hydrolysis (6 N HCl, 100 °C, 24 h in a sealed tube) of the isolated putative mediator followed by carbohydrate analysis on Dionex HPLC demonstrated that chiro-inositol was the sole inositol present. Analysis on a chiral GC column determined that the inositol was solely D-chiro-inositol; no L-chiro-inositol was detected. To prevent degradation of the amino sugar, acid hydrolysis was performed under less vigorous conditions (4 N trifluoroacetic acid, 100 °C, 4 h). Surprisingly, pinitol **2b**, the 3-*O*-methyl ether of D-chiro-inositol, was detected together with galactosamine in a molar ratio of 1.2:1. No free D-chiro-inositol was detected. No significant amounts of other sugars were present.

Nitrous acid oxidative deamination followed by reduction with sodium borotritiide generated 2,5-[^{3}H]anhydrotalitol, clearly distinguished by HPLC from [^{3}H]-anhydromannitol, the expected product from glucosamine. In the homogeneous material, only traces of inorganic and no organic P was detected. Thus, chemical studies indicated the presence of pinitol linked to galactosamine as a non-phosphorylated pseudo-disaccharide (Figure 1).

NMR Structure Determination. The structure of the pseudo-disaccharide was established by 2D nuclear magnetic resonance spectroscopy to be 4-*O*-(2-amino-2-deoxy-β-D-galactopyranosyl)-3-*O*-methyl-D-chiro-inositol **1**. Assignment of proton and carbon resonance frequencies on each saccharide moiety was accomplished by analysis of double quantum filtered correlation (DQCOSY), homonuclear Hartmann−Hahn (HOHAHA), and heteronuclear proton−carbon multiple quantum correlation (HMQC) spectra. From the spectra, the organic purity of the sample was high (>98%).

The determination of the stereochemistry of the linkage was based on the observed coupling constant between H1 and H2 of galactosamine.[9a] The large coupling constant (J = 8.5 Hz) indicated that the glycosidic linkage was β as opposed to α. The location of the linkage on pinitol was determined by the large chemical shift change for C4′ of pinitol[9b] and the existence of a large proton−proton nuclear Overhauser effect between H1 of galactosamine and H4′ of pinitol.

Chemical Synthesis. Pseudo-disaccharide **1** was prepared employing the trichloroacetimidate method (Scheme 1).[10] Neighboring group participation of 2-*N*-acyl-protecting groups of glycosyl donors is usually the dominating effect in controlling formation of the anomeric stereocenter in the synthesis of aminoglycosides using this method.[10] To install the requisite β-1,2-trans linkage of the pseudo-disaccharide, a number of amino protecting groups were investigated. The trichloroethoxycarbonyl (Troc) group was selected on the basis of superior β/α selectivity as well as ease of removal. This glycosyl donor **5** was readily available from galactosamine in multigram quantities, requiring only one chromatographic step. The glycosyl acceptor **6** was prepared from pinitol **2** in one step. Trimethylsilyl triflate assisted glycosidation gave the desired β-anomer **7** and its chromatographically separable α-anomer (ratio 9:1). A convenient two-step deprotection sequence involving acid hydrolysis of the dimethyl ketals followed by mild base hydrolysis of the Troc and acetate residues completed the synthesis of the glycan component of **1** in 18% overall yield from galactosamine.

A comparison by ^{1}H NMR of synthetic and natural **1** indicated subtle differences between the two spectra. It is known that chemical shifts of amino sugars are sensitive to pH. Pseudo-disaccharide from beef liver was isolated under acidic conditions, while the synthetic pseudo-disaccharide was obtained as the free amine. When synthetic pseudo-disaccharide was acidified by the addition of CF_3COOD in D_2O, the ^{1}H NMR spectrum was identical in all respects with that of the natural material, confirming the structure of the glycan component of the putative mediator (see Supporting Information for ^{1}H NMR spectra of natural and synthetic **1**).

A sample of synthetic pseudo-disaccharide **1** was hydrolyzed as for the isolated putative mediator (6 N HCl, 100 °C, 24 h in a sealed tube). Carbohydrate analysis on Dionex HPLC demonstrated chiro-inositol as the sole inositol present, confirming that no isomerization to other inositols occurs and also that pinitol is demethylated under these hydrolysis conditions.

Studies on the Metal Chelated Structure. Significant line broadening was observed in the ^{1}H NMR of freshly prepared beef liver pseudo-disaccharide samples. Over time and in solution, this disappeared, suggesting a paramagnetic material complexed with the pseudo-disaccharide. Analysis by nuclear activation analysis (NAA) indicated the presence of manganese in the ratio of approximately 4:1 (Mn/pseudo-disaccharide). Analysis of the beef liver pseudo-disaccharide by electron paramagnetic resonance spectroscopy (EPR) demonstrated at 77 K a multiline spectrum at about $g = 2$ indicative of Mn in its +2 oxidation state (Mn^{3+} is an EPR-silent species).[11]

It is known from chelation studies of aminomonosaccharides that the primary site of metal chelation occurs at the amino functionality. Further, amino sugars chelating paramagnetic metal ions exhibit line broadening in both their ^{1}H and ^{13}C NMR spectra at positions proximal to the metal.[12] Mn^{2+} binding to the synthetic pseudo-disaccharide was investigated by acquiring 2D carbon−proton HMQC spectra at various concentrations of Mn^{2+} (ratio of Mn^{2+} to pseudo-disaccharide of 6×10^{-4} to 2.5×10^{-3}). HMQC spectra were used for this purpose to remove spectral overlap. The largest line broadening occurred at H2 of galactosamine, compatible with chelation by the amino nitrogen. Broadening also occurred at H3 and H1 although at lower magnitude. On pinitol, broadening was observed at H4′, H5′, and H6′. No broadening was observed at H5, H6, H3′, or OCH_3. This suggests that chelation occurs regioselectively at the front face of the pseudo-disaccharide, as shown in Figure 1.

Synthetic pseudo-disaccharide Mn^{2+} chelate samples mimicking the pharmacological effects of the natural material could be prepared by treating the synthetic pseudo-disaccharide with $MnCl_2$ (Scheme 1). An aqueous solution of synthetic glycan was adjusted to pH 6.8 by the addition of HOAc. The solution was concentrated under vacuum, and 0.40 M aqueous $MnCl_2$ was added. This solution was concentrated, and the material was used in vitro.

In Vivo and in Vitro Pharmacology. Low-dose STZ (*N*-[methylnitrosocarbamoyl]-D-glucosamine) diabetic animals were anesthetized with ketamine (2-[2-chlorophenyl]-2-[methylamino]cyclohexanone), and zero time plasma glucose was determined by tail vein sampling. Synthetic unchelated pseudo-disaccharide (INS-2) dissolved in saline was then injected intravenously (tail vein), and blood glucose was determined at time intervals as shown. Control diabetic animals were also injected with saline. Synthetic INS-2 injected intravenously into hyperglycemic STZ diabetic rats decreased elevated blood glucose at a dose of 2 mg/kg (6 mM). A 40% statistically significant decrease was observed at 30−45 min with INS-2 (Figure 4). The α-anomer at the same dose did not produce a statistically significant effect.

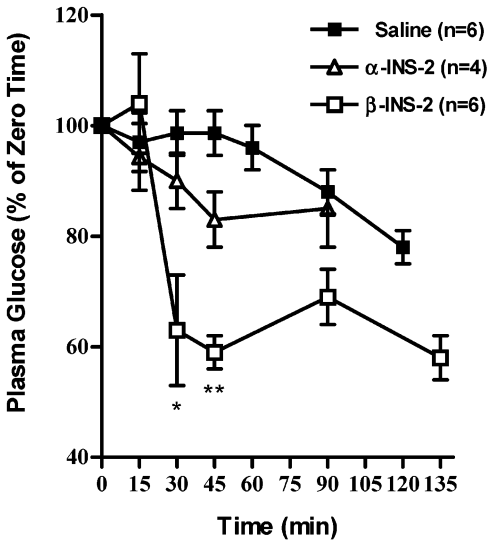


Figure 4. Anomeric specificity of α- and *β*-INS-2 to decrease hyperglycemia in STZ diabetic rats in vivo. STZ diabetic rats (Sprague−Dawley) were anesthetized with ketamine, and zero time blood samples were obtained by tail clipping. α- and *β*-INS-2 (2 mg/kg), or saline, were separately injected into tail veins as a bolus, and blood sugars were analyzed at the indicated times. The number of animals are as indicated. (*) Statistically significantly different from control, $p < 0.006$. (**) $p < 0.001$.

Unchelated INS-2 was injected into STZ diabetic rats in the dose range of 2−20 mg/kg in an independent set of experiments (Figure 5). All three doses of INS-2 reduced blood glucose in a statistically significant manner at 100 and 120 min. At 20 mg/kg, elevated blood glucose was statistically significantly reduced at all time points. The largest effect in reducing hyperglycemia occurred at 40, 60, and 80 min with the dose response of the drug being statistically significant at 60 min. At a dose of 20 mg/kg (60 μM), about a 40% decrease was observed.

When tested on insulin-sensitive hepatoma H4IIE cells in the presence of insulin, unchelated INS-2 further stimulated [^{14}C]glucose incorporation into glycogen in a dose-dependent manner (Figure 6). INS-2 was effective at 0.1−10 μM with a maximal effect at 100 μM. In the absence of insulin, INS-2 was inactive. In an additional experiment (data not shown), the Mn^{2+}/INS-2 chelate (4:1 and also ratios down to INS-2 alone) was also equally effective in the presence of insulin.

The Mn^{2+}/INS-2 chelate (4:1) stimulated PDH phosphatase maximally in vitro above an equivalent concentration of free Mn^{2+} in the 100 μM range (Figure 7). Maximal velocity was statistically increased, as was

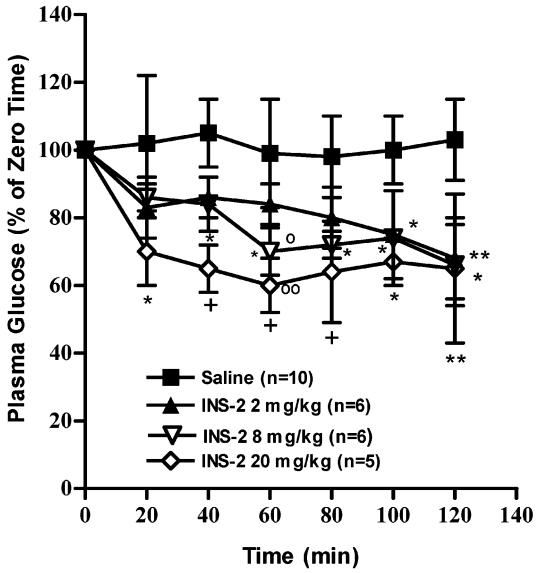


Figure 5. Dose−response of INS-2 to decrease hyperglycemia in STZ diabetic rats in vivo. STZ diabetic animals (Wistar) were anesthetized with pentobarbital (50 mg/kg) and injected with propranolol ip (1 mg/kg) to minimize sympathetic stimulation. Animals were separately injected with INS-2 as a bolus (2, 8, or 20 mg/kg or saline) into the jugular vein, and blood sugars from the tail vein were analyzed at the indicated times. The number of animals are as indicated. (∗) Statistically different from control, $p < 0.05$. (∗∗) $p < 0.001$. (+) $p < 0.01$. (○) Significantly different from 2 mg/kg at 60 min, $p < 0.05$. (○○) Significantly different from 8 mg/kg at 60 min, $p < 0.05$. ANOVA followed by Bonferroni was used.

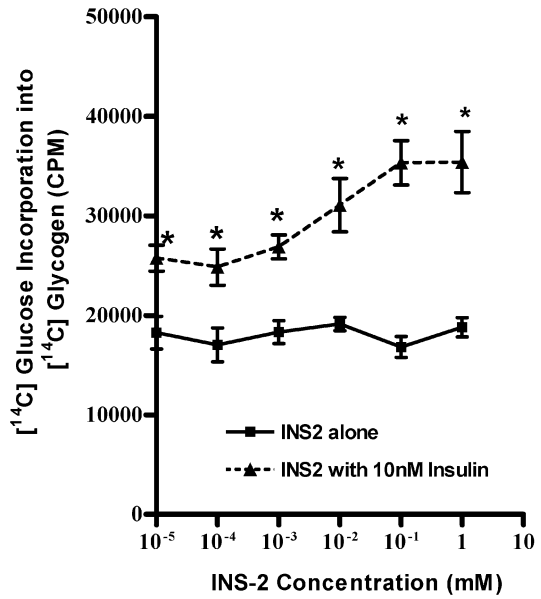


Figure 6. Dose−response of INS-2 enhancing insulin action to stimulate [^{14}C]glycogen synthesis in H4IIE hepatoma cells. Hepatoma cells were treated with INS-2 in the presence of insulin or INS-2 alone for 60 min at 37 °C in the presence of [^{14}C]glucose (2 μCi) per cell. Cells were washed and glycogen was isolated and counted following cell digestion with KOH as described in Experimental Section. Note the micromolar dose−response of INS-2 in the presence of insulin to stimulate glycogen synthesis in contrast to the lack of action of INS-2 alone. Number of experiments is 3. (∗) Statistically different from control, $p < 0.005$.

sensitivity, as indicated by the left shift of the concentration curve. INS-2 alone was completely inactive.

Discussion

A novel β-galactosamine-D-chiro-inositol pseudodisaccharide Mn^{2+}chelate was isolated from fed beef liver. Its structure was established by chemical degradation, 2D NMR, and chemical synthesis (Figure 1, Scheme 1). Several unique structural features require comment. First, the presence of D-chiro-inositol linked to galactosamine is novel. D-chiro-Inositol and its 3-*O*-methyl ether, pinitol **2**, were previously known only in plants and insects.[13] Recently, several reports documented chiro-inositol including D- and L-chiro-inositol together with myo-Inositol in tissue hydrolysates from animal sources.[14] Pinitol, however, has been reported in plants[13] but not in animal materials. The *O*-methyl ether is likely important for directing stereospecific metal binding, since from model studies it is clear that methylation of carbohydrate hydroxyl groups prevents them from participating in metal chelation.[15] Second, the presence of a β-1,4 linkage between pinitol and galactosamine is novel because all naturally occurring glycosylphosphatidylinositol structures thus far studied have an α-1,6 linkage between glucosamine and myo-inositol (Figure 2).[16] Third, the presence of chelated transition metal in an inositol glycan natural product is novel, although the literature has provided examples of simple model mono- and disaccharide metal chelates.[17] Synthetic unchelated INS-2 in vivo reduced hyperglycemia in low-dose STZ diabetic rats, a model of type 2 diabetes (Figures 4 and 5). Stereoselectivity was demonstrated with the β-anomer active and the

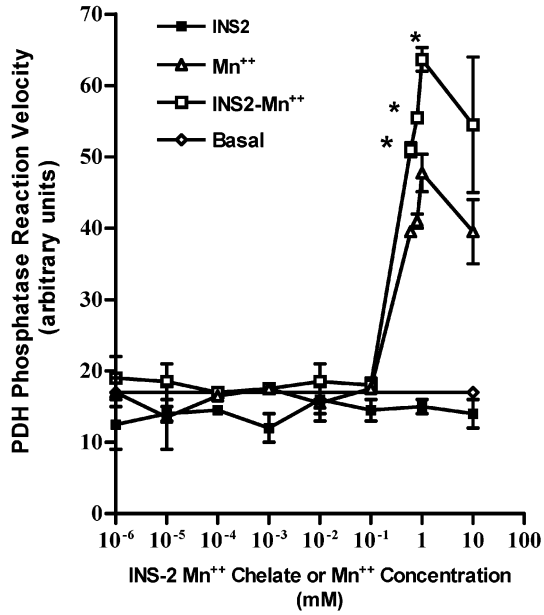


Figure 7. Mn^{2+} chelate of INS-2 activates PDH phosphatase in vitro. Mn^{2+} alone and Mn^{2+} chelate of INS-2 (4:1) were incubated with PDH and PDH phosphatase for 30 min. Note the activation of PDH phosphatase above that of Mn^{2+} alone. Note also the lack of effect of INS-2 alone. The mean is from four experiments. (∗) Statistically different from control, $p < 0.02$.

α-anomer inactive at a dose of 2 mg/kg (Figure 4). In vivo unchelated INS-2 produced a dose responsive decrease in hyperglycemia in low-dose STZ diabetic rats between 2 and 20 mg/kg (Figure 5). In rat hepatoma cells, it enhanced [^{14}C]glucose incorporation into [^{14}C]-

glycogen in a dose-dependent manner only in the presence of insulin (Figure 6). Thus, in this model cell system, it enhanced insulin action. In vitro, in a defined enzyme assay, only the Mn^{2+} chelate was effective to activate PDH phosphatase. Here, it both sensitized the enzyme to Mg^{2+} and increased its maximum velocity (Figure 7). INS-2 alone was inactive. Presumably in intact cells and in vivo, INS-2 is able to chelate a transition metal (Mg^{2+} or Mn^{2+}) to effect biological activity. Alternatively, in vivo INS-2 alone may be inherently active.

Independently, Nestler et al. demonstrated the insulin mimetic properties of synthetic INS-2.[18] When human ovarian thecal cells were treated with insulin or INS-2, they were equally effective in stimulating maximal testosterone production. INS-2 was less potent because it acted at micromolar concentrations in contrast to insulin, which acted at nanomolar concentrations. That INS-2 was indeed acting in a similar mechanistic manner as insulin was evidenced by the fact that both its action and that of insulin were blocked by a specific antibody raised against INS-2.[18] *In summary, results from this and several other laboratories demonstrate that this present pseudo-disaccharide is a putative insulin mediator acting at micromolar concentrations in vivo alone and in vitro on PDH phosphatase only as a chelate.*

The novel structure of INS-2 suggests a novel lipid or proteinated GPI precursor distinct from known sequenced species. Chiro-inositol containing lipid species have been identified in the literature.[14c,19] Specifically, Pak and Larner have identified four GPI lipids with 1:1 molar ratios of chiro-inositol to galactosamine.[19] Further, Pak et al. demonstrated that addition of insulin to a rat fibroblast line expressing the human insulin receptor stimulates the epimerization of myoinositol to chiro-inositol phospholipids, thus enhancing their synthesis.[20]

Evidence of cleavage of precursors to inositol glycans by insulin stimulation has also been presented. Experiments have documented the release of inositol glycans from rat liver membranes upon addition of insulin.[21] Released glycans were determined chemically after strong acid hydrolysis as free inositols[21b] and by bioactivity to stimulate PDH phosphatase (see Figure 7).[21a] Further, the PDH phosphatase stimulatory activity was identified as an inositol glycan by its immunoprecipitation by a specific IgG antibody.[21a] We have shown that this occurs via a heterotrimeric G protein linked pathway[21a] with the G protein recently identified as Gq.[21b] Thus, the total evidence suggests, but does not prove, that INS-2 is present in fed (insulinized) beef liver as a result of processing from a larger precursor.

Consistent with the involvement of a precursor to INS-2, this laboratory previously purified a chiro-inositol containing beef liver fraction that was even more active as an insulin mimetic than INS-2. When perfused into low-dose STZ diabetic animals, it decreased hyperglycemia to euglycemia at nanomolar concentrations (chiro-inositol) similar to those of insulin.[8b] The relationship of INS-2 to the more active chiro-inositol species is under current investigation.

In a series of elegant experiments, Müller and colleagues have purified a GPI anchored protein from yeast, termed Gce1p, which binds cAMP and which is released into the medium on the addition of sulfonylurea.[5b,22] Following cleavage by V8 protease and phosphatidylinositol specific phospholipase C, the isolated core IPG linker glycopeptide was demonstrated to be an insulin mimetic with glycan structure, **3a** (Figure 2).[5b] To determine the structural features necessary for activity, a number of IPG compounds were synthesized by this group, including molecules with the conserved and shortened (or mutated) glycan core.[6d] These compounds were tested for insulin-mimetic effects on key metabolic pathways of lipogenesis, glycogenesis, and lipolysis. Surprisingly, although the α-linked IPGs were active, derivatives possessing the *β*-glucosamine myo-inositol polar headgroup, as in **4** (byproducts of the glycosidation step) were significantly more active. Thus, the enhanced bioactivity of the synthetic *β*-linked glycans over the α-linked glycans is in keeping with the stereospecificity noted in our experiments (Figure 4). Although Müller et al. tested INS-2, they did not report in vivo experiments to determine its efficacy to decrease hyperglycemia in diabetic animal models.

A recent report by Martín-Lomas, Rademacher, and co-workers[6c] demonstrated that compounds such as **3b** did not possess insulin-like activity in two enzyme-based assays (stimulation of PDH phosphatase or inhibition of cAMP kinase) and concluded that traditional GPI anchors are thus unlikely precursors of insulin mimetic IPGs. Certainly more extensive bioassays of in vitro and in vivo insulin mimetic effects will be required to rule out bioactivity in view of the extensive data of Müller and collaborators.

In summary, one may deduce that our *β*-linked pseudo-disaccharide arises from a novel *β*-linked precursor, i.e., a GPI lipid and/or a proteinated species.

Added Note. A report has appeared demonstrating the conversion of myo-Inositol to chiro-inositol in GPI anchored proteins as an artifact of acid catalysis.[23] The absolute configuration of the chiro-inositol produced was not determined. These authors questioned the validity of much of our analytical work on D-chiro-inositol.

The authors state "isomerization occurred during hydrolysis of the GPI anchor, whose inositol is linked to glucosamine and phosphate at 6-OH and 1-OH, respectively".[23] Thus, for the artifactual isomerization of myoinositol to chiro-inositol to occur under the stated acidic conditions, both glucosamine and covalently bound phosphate were necessary. Since neither the beef liver nor the chemically synthesized *β*-pseudo-disaccharide contained myo-Inositol, organic P, or glucosamine, it would appear unlikely that the present D-chiro-inositol results can be ascribed to the isomerization mechanism demonstrated in the acid hydrolysis of GPI anchored proteins.

Furthermore, from the present and previously published data from this lab[14a] and from other labs,[14b] the presence of predominately free D-chiro-inositol (minimal L-chiro-inositol) in urine and blood has been quantified under nonacid hydrolyzed conditions. The concentration of D-chiro-inositol is inversely related to the degree of insulin resistance in Rhesus monkeys[24] and humans.[25] Thus, while caution must be exercised in evaluating chiro-inositol data after strong acid hydrolysis of natural materials, the totality of the present and past data

strongly supports the proposed role for a relationship of free urine D-chiro-inositol in insulin resistance and a role for the present pseudo-disaccharide conjugate of D-chiro-inositol as an insulin mimetic.

Experimental Section

Isolation and Pharmacology. Pyruvate dehydrogenase phosphatase was assayed as previously described.[2,3] One unit of PDH phosphatase was defined as the difference between activity at low Mg^{2+} subtracted from the activity at high Mg^{2+} under the stated conditions. Chiro-inositol was analyzed by Dionex HPLC as previously described after hydrolysis in sealed tubes in 6 N HCl for 24−48 h.[4] Standard D- and L-chiro-inositol were analyzed on a chiral column with 0.3 min baseline resolution as follows. Samples were dried in vacuo to complete dryness. A total of 100 μL of HFBI (hexafluorobutyrylimidazole) was added, and samples were incubated at 60 °C overnight. Samples were extracted into 200 μL of hexane and vortexed. Then an amount of 200 μL of H_2O was (deionized and glass-distilled) added and vortexed, and the hexane layer was removed. The H_2O layer was washed twice with hexane. Hexane washes were combined and concentrated under vacuum to dryness. Samples were dissolved in 1 mL of hexane, and 1 μL was injected onto a 25 m Chirasil-Val (Alltech) GC capillary column. Column temperature was set at 90 °C for 4 min and then ramped at 25 °C/min to 130 °C. The column temperature was then ramped to 180 °C and held for 5 min. Detection was by FID using the retention time of standards as a guide.

Male Sprague−Dawley rats were injected intravenously with low-dose streptozotocin (50 mg/kg) to induce type 2 diabetes. After 7 days, diabetes was established by tail vein blood glucose values measured in the range of 250−400 mg/100 mL. INS-2 and its α-isomer were injected in the tail vein of animals anesthetized with ketamine, and blood samples were collected at indicated times (Figure 4). In an independent set of experiments conducted in Brazil (Figure 5), Wistar rats were injected with streptozotocin (50 mg/kg), and after 2 days, diabetes was established by tail vein blood glucose analysis, which again measured in the range 250−400 mg/100 mL as above with a mean of 343 ± 50 mg/100 mL. To establish a dose−response relationship, three concentrations of INS-2 were injected via the jugular vein into pentobarbital (5-ethyl-5-[1-methylbutyl]-2,4,6-trioxohexahydropyrimidine) anesthetized (50 mg/kg) animals previously injected with propranolol (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanol hydrochloride) (1 mg/kg) intraperitoneally to avoid sympathetic stimulation. Serial blood samples were withdrawn for blood glucose analysis from the tail vein at the indicated times. Blood glucose was determined with a glucometer assay.

H4IIE hepatoma cells were grown in HAM's F-12/DMEM (1:1) with 10% fetal bovine serum containing 5 mM glucose in plates containing 24 wells. Prior to testing, the medium was replaced in the evening with HAM's F-12/DMEM (1:4) with 0.1% fetal bovine serum and 2 mM glucose and cells were incubated overnight. In the morning, the medium was removed and cells were washed two times with PBS. Cells were incubated in PBS with [^{14}C]glucose, 0.2 μCi per well, and unlabeled 100 μM glucose was added. Incubation was for 60 min at 37 °C. At end of incubation, cells were washed two times with PBS, 100 μL of 30% KOH was added, the sample was incubated for 60 min at 37 °C, the contents were removed and heated at 100 °C for 15 min, and glycogen was precipitated with four volumes ethanol. After the mixture stood overnight, glycogen was recovered by centrifugation and pellets were washed with 70% ethanol, dissolved in H_2O, and counted in the scintillation counter.

Statistics. Two-tailed paired *t*-test was used to determine the mean, the standard error of the mean, and *p* values using GraphPad Prizm version 3.02 for Windows, GraphPad Software, San Diego, California, www.graphpad.com. ANOVA followed by Bonferroni was used when comparing more than two mean values.

Chemistry. NMR spectra were recorded at 30 °C on a Varian XL-500 (500 MHz) instrument with TMS as reference. Optical rotations were determined with a Jasco DIP-360 polarimeter at ambient temperature. Elemental analyses were obtained from Galbraith Laboratories. Highly accurate mass spectrometry was obtained using a Micromass LCT mass spectrometer. Thin-layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminum sheets, silica 60). Products were visualized by spraying with phosphomolybdic acid in methanol, followed by heating. Separation and purification of products were performed by flash chromatography using silica gel Merck (230−400) mesh by the method developed by Still and co-workers.[26] Chemicals and anhydrous solvents were purchased from Sigma-Aldrich and used without further purification. Pinitol was obtained from New Zealand Pharmaceuticals.

3-*O*-Methyl-1,2:5,6-bis-*O*-(1-methylethylidene)-D-chiro-inositol (6). To a suspension of 10.0 g (51.5 mmol) of D-pinitol **2b** in DMP/acetone (200 mL, 1:3) was added pTsOH (380 mg, 2.0 mmol). The mixture was stirred at room temperature for 12 h, after which a homogeneous solution was obtained. Solid $NaHCO_3$ (1.00 g, 11.9 mmol) and then solid $MgSO_4$ were added. The mixture was filtered and concentrated under reduced pressure to afford 12.2 g (44.4 mmol, 87%) of **6**. This material could be used without further purification. An analytical sample was obtained by flash chromatography (EtOAc/hexane 1:1, R_f = 0.38). Spectral data were in agreement with literature values.[27]

1-Trichloroacetimido-2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-α-D-galactopyranose 3,4,6-Triacetate (5). To a solution of of galactosamine hydrochloride (6.00 g, 27.8 mmol) in H_2O (60 mL) at 0 °C was added 5.84 g (69.5 mmol) of $NaHCO_3$. The solution was stirred for 10 min, and 5.1 mL (7.84 g, 37.0 mmol) of TrocCl was added dropwise over 5 min. The mixture was allowed to warm to room temperature and stirred for 10 h. A white precipitate formed. The suspension was cooled to 0 °C, and the precipitate was collected by filtration, washed with Et_2O, and vacuum-dried.

This material was stirred overnight in 120 mL of a mixture of pyridine/Ac_2O (1:1). The reaction mixture was concentrated at room temperature, poured into cold water (500 mL), and extracted with EtOAc (3 × 200 mL). The combined organic extracts were washed with H_2O (3 × 200 mL), 1 N aqueous HCl (200 mL), and saturated aqueous $NaHCO_3$ (200 mL), dried over $MgSO_4$, concentrated, and dried under vacuum to afford 11.3 g (21.6 mmol) of 2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-D-galactopyranose 1,3,4,6-tetraacetate (mixture of α- and β-anomers) as a colorless glassy solid suitable for use in the next step.

A solution of the tetraacetate (11.3 g, 21.6 mmol) and hydrazine acetate (2.29 g, 24.8 mmol) in anhydrous DMF (75 mL) was stirred under Ar at room temperature overnight. The reaction mixture was concentrated under reduced pressure at 60 °C and poured into Et_2O (500 mL). The organic fraction was washed with H_2O (2 × 500 mL), dried over $MgSO_4$, and concentrated under reduced pressure to afford 6.92 g (14.4 mmol) of 2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-D-galactopyranose 3,4,6-triacetate. To a solution of the triacetate (6.92 g, 14.4 mmol) in anhydrous CH_2Cl_2 (50 mL) was added 1.45 mL (2.08 g, 14.4 mmol) of Cl_3CCN and 322 μL (330 mg, 2.16 mmol) of DBU. The mixture was stirred at room temperature overnight and concentrated under reduced pressure. The residue was promptly purified by flash chromatography (EtOAc/hexane 1:3, 275 g of silica gel) to afford **5** as a white glassy solid (5.81 g, 9.31 mmol, 34% from galactosamine, >95% α-anomer by ^{1}H NMR): $[\alpha]^{23}_D$ +62.7° (*c* 0.0100 g/mL, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.80 (s, 1H), 6.46 (d, J = 3.0 Hz, 1H), 5.51 (br s, 1H), 5.28 (dd, J = 11.5, 2.5 Hz, 1H), 5.12 (d, J = 9.5 Hz, 1H), 4.76 (d, J = 12.0 Hz, 1H), 4.70 (d, J = 12.0 Hz, 1H), 4.53 (m, 1H), 4.38 (m, 1H), 4.17 (m, 1H), 4.08 (m, 1H), 2.19 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 170.1, 170.0, 160.3, 154.3, 95.2, 95.0, 90.6, 74.5, 68.9, 67.7, 66.4, 61.0, 49.3, 20.5 (3C)) ppm.

4-[2-Deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-3,4,6-triacetyl-β-D-galactopyranosyl]-3-*O*-methyl-1,2:5,6-bis-*O*-(1-methylethylidene)-D-chiro-inositol (7). Molecular

sieves (4 Å, 2.5 g) were vacuum-dried at 150 °C for 10 h and then cooled and suspended with stirring in anhydrous CH_2Cl_2 (15 mL) under argon. To this suspension were added trichloroacetimidate **5** (0.50 g, 0.80 mmol) and diacetonide **6** (0.21 g, 0.765 mmol), both azeotroped with anhydrous benzene (2 × 25 mL). The suspension was stirred for 30 min at room temperature, cooled to −25 °C, and treated with $CF_3SO_2SiMe_3$ (1.92 mL of a 0.42 M solution in toluene, 0.80 mmol). The mixture turned light-purple, was allowed to warm to room temperature, and was stirred overnight. The reaction mixture was filtered through Celite, and the filter cake was washed with CH_2Cl_2 (2 × 25 mL). The combined filtrate and washings were washed with saturated aqueous $NaHCO_3$ (100 mL), dried over $MgSO_4$, and concentrated at reduced pressure. The residue was purified by flash chromatography (EtOAc/hexane 2:3, 15 g of silica gel) to afford **7** as a colorless glassy solid (377 mg, 0.51 mmol, 67%): $[\alpha]^{23}_D$ +13.8° (*c* 0.0442 g/mL, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.35 (s, 1H), 5.27 (d, J = 9.5 Hz, 1H), 4.88 (d, J = 8.0 Hz, 1H), 4.73 (br s, 1H), 4.30−4.10 (m, 7H), 3.98 (m, 1H), 3.88 (t, J = 6.5 Hz, 1H), 3.77 (dd, J = 10.5, 7.0 Hz, 1H), 3.58 (s, 3H), 3.22 (m, 1H), 2.16 (s, 3H), 2.03 (s, 3H), 1.94 (s, 3H), 1.52 (s, 3H), 1.51 (s, 3H), 1.36 (s, 3H), 1.35 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 170.4, 170.3, 154.3, 109.8, 109.5, 100.9, 95.6, 80.0, 79.8, 79.1, 78.2, 76.1, 75.8, 74.3, 70.8, 70.7, 66.4, 61.2, 60.2, 53.0, 27.8, 27.7, 25.3 (3C), 20.6 (2C) ppm.

4-(2-Amino-2-deoxy-*β*-D-galactopyranosyl)-3-*O*-methyl-D-chiro-inositol (INS-2, 1). A solution of fully protected INS2 (**7**, 4.07 g, 5.31 mmol) in 80% AcOH (50 mL) was heated at 80 °C for 5 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (MeOH/$CHCl_3$ 1:9, 80 g of silica gel) to afford 2.94 g of a colorless solid. This material was dissolved in 2-propanol/H_2O (120 mL, 1:5), and Amberlite 420 resin (Cl form, 45 g, washed with 10 volumes of distilled deionized (DDI) H_2O, 5 volumes of 2-propanol, 10 volumes of 1 N NaOH, and 20 volumes of DDI H_2O until neutral) was added. The suspension was heated at 65 °C for 3 h. The mixture was cooled, filtered, and concentrated under reduced pressure to $^1/_3$ of the initial volume. Filtration through Whatman 42 paper and concentration afforded **1** as a colorless solid (1.47 g, 4.14 mmol, 78%): $[\alpha]^{23}_D$ +43.8° (*c* 0.0295 g/mL, H_2O); ^{1}H NMR (D_2O, pH 7.8, 500 MHz) δ 4.47 (d, J = 8.5 Hz, 1H, H-1), 3.88−3.85 (m, 2H), 3.82 (dd, J = 10.0, 2.8 Hz, 1H), 3.77−3.67 (m, 4H), 3.63 (dd, J = 12.0, 4.5 Hz, 1H), 3.53 (ddd, J = 7.5, 4.5, 1.0 Hz, 1H, H-5), 3.47 (s, 3H, OCH_3), 3.44 (dd, J = 10.0, 3.3 Hz, 1H), 3.31 (t, J = 9.5 Hz, 1H, H-3′), 2.73 (dd, J = 10.0, 8.5 Hz, 1H, H-2); ^{13}C NMR (D_2O, 125 MHz) δ 104.2, 80.5, 80.3, 74.8, 72.7, 71.0, 70.9, 70.3, 69.2, 67.5, 60.8, 58.9, 53.5 ppm. Exact mass calculated for $C_{13}H_{26}NO_{10}$ ($M + H^+$) 356.1557, found 356.1587. Anal. ($C_{13}H_{25}NO_{10}\cdot xH_2O$): C, calcd 42.86, found 42.58; H, calcd 7.19, found 7.41; N, calcd 3.84, found 3.78.

Preparation of INS-2/Mn^{2+} Chelate. An aqueous solution of INS-2 (36 mg, 0.10 mmol, 20 mM) was adjusted to pH 6.8 by the addition of 100 mM aqueous HOAc. The solution was concentrated under vacuum, and 1.00 mL of 0.40 M $MnCl_2$ (0.40 mmol) was added. This solution was concentrated, and the material was used in vitro.

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Supporting Information Available: A figure comparing the ^{1}H spectra of the acidic (isolated) and neutral (synthetic) forms of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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