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Deoxygenated phosphorothioate inositol phosphate analogs: Synthesis, phosphatase stability, and binding affinity

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Abstract—Inositol phosphates, such as 1D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], are cellular second messengers with potential roles in cancer prevention and therapy. It typically is difficult to attribute specific pharmacological activity to a single inositol phosphate because they are rapidly metabolized by phosphatases and kinases. In this study, we have designed stable analogs of *myo*-inositol 4,5-bisphosphate [Ins(4,5)P_2] and Ins(1,4,5)P_3 that retain the cyclohexane scaffold, but lack hydroxyl groups that might be phosphorylated and have phosphate groups replaced with phosphatase-resistant phosphorothioates. An Ins(1,4,5)P_3 analog, 1D-2,3-dideoxy-*myo*-inositol 1,4,5-trisphosphorothioate, was synthesized from (-)-quebrachitol, and an Ins(4,5)P_2 analog, 1D-1,2,3-trideoxy-*myo*-inositol 4,5-bisphosphorothioate, was prepared from cyclohexenol. The Ins(1,4,5)P_3 analog was recognized by Ins(1,4,5)P_3 receptor with a binding constant (K_d) of 810 nM, compared with 54 nM for the native ligand Ins(1,4,5)P_3, and was resistant to dephosphorylation by alkaline phosphatase under conditions in which Ins(1,4,5)P_3 is extensively hydrolyzed. Analogs developed in this study are potential chemical probes for understanding mechanisms of inositol phosphate actions that may be elucidated by eliciting specific and prolonged activation of the Ins(1,4,5)P_3 receptor.

1. Introduction

Myo-Inositol and its corresponding hexaphosphate ($InsP_6$, Chart 1) are widely distributed in plants and animals. Both have been shown to possess cancer preventive and therapeutic activity in vitro and in animal studies.[1–8] In chemoprevention studies, these compounds were effective in reducing tumor number and size when given simultaneously with or after carcinogen exposure.[4–8] The biochemical mechanisms underlying the chemopreventive activity of inositol compounds are not known, but exogenously administered $InsP_6$ is rapidly dephosphorylated to lower inositol phosphates that participate in signal transduction pathways and affect the cell cycle.[9] Preclinical studies indicate that the combination of inositol with $InsP_6$ further enhances chemopreventive and anticancer effects and it has been suggested that this combination disproportionates to Ins(1,4,5)P_3, which normalizes the rate of cell division thus preventing tumor formation. The resulting hypothesis is that inositol phosphates with fewer than six phosphate groups, such as the biochemical second messenger Ins(1,4,5)P_3, may be responsible for the biological activities observed for inositol and $InsP_6$.[10–13]

There have been extensive studies of the roles of inositol and inositol phosphates in signal transduction and of the enzymes that catalyze the conversions between different inositol phosphates.[14–21] Among the various inositol phosphates, Ins(1,4,5)P_3 is the most understood; it is established as an important second messenger involved in many signaling pathways. After cleavage from the membrane-bound phosphatidylinositol trisphosphate, Ins(1,4,5)P_3 binds to its corresponding receptor and stimulates the release of stored Ca^{2+} into the cytoplasm. This event triggers an array of cellular responses including glycogen breakdown, muscle contrac-

Abbreviations: Ins, *myo*-inositol; $InsP_1$, *myo*-inositol-monophosphate; $InsP_2$, *myo*-inositol-bisphosphate; Ins(4,5)P_2, 1D-*myo*-inositol 4,5-bisphosphate; Ins(1,4,5)P_3, 1D-*myo*-inositol 1,4,5-trisphosphate; $InsP_6$, *myo*-inositol hexaphosphate; K_d, dissociation constant; CID, collision-induced dissociation; DTT, DL-dithiothreitol; MCPBA, *meta*-chloroperbenzoic acid; AMP, adenosine 2′-monophosphate; AMPS, adenosine 5′-*O*-thiomonophosphate; TEAB, triethylammonium bicarbonate; MS, mass spectrometry; *p*NPP, *p*-nitrophenyl phosphate.

Keywords: *Myo*-inositol 1,4,5-trisphosphate; Ins(1,4,5)P_3 analog; Binding affinity; Ins(1,4,5)P_3 receptor; Phosphatase-stable analogs.

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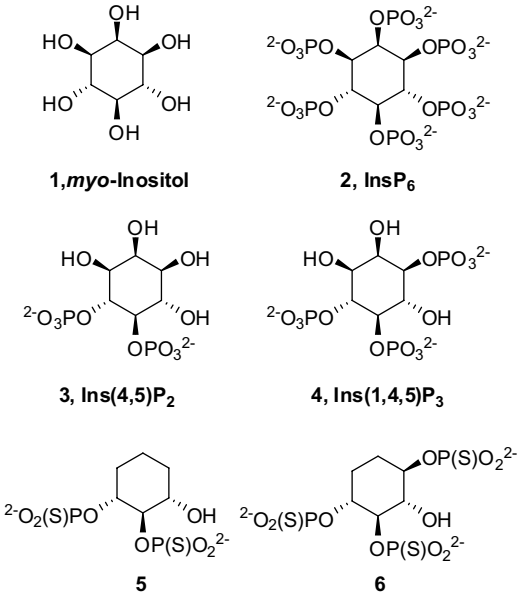


Chart 1. Structures of D-*myo*-inositol (**1**), D-*myo*-inositol hexaphosphate (**2**), 1D-*myo*-inositol 4,5-bisphosphate (**3**), and 1D-*myo*-inositol 1,4,5-trisphosphate (**4**), 1D-1,2,3-trideoxy-*myo*-inositol 4,5-bisphosphorothioate (**5**), 1D-2,3-dideoxy-*myo*-inositol 1,4,5-trisphosphorothioate (**6**).

tion, and the release of neurotransmitters.[22–24] $Ins(1,4,5)P_3$ is rapidly deactivated by stepwise dephosphorylation to $Ins(1,4)P_2$, then to Ins-4-P_1 and finally *myo*-inositol.[25,26] Alternatively, phosphorylation by $Ins(1,4,5)P_3$ 3-kinase produces $Ins(1,3,4,5)P_4$.[27,28] The metabolic instability and rapid clearance of $Ins(1,4,5)P_3$ make it difficult to probe directly the role of $Ins(1,4,5)P_3$ in biological events, such as those that may underlie mechanisms of inositol and $InsP_6$-mediated cancer chemoprevention. Thus, the overall benefits of $Ins(1,4,5)P_3$ in cancer prevention and therapy remain controversial.

Inositol phosphate chemical analogs resistant to enzymatic degradation have potential for enhanced chemopreventive activity and mechanistic information obtained could serve as a starting point for medicinally focused approaches to develop improved agents for chemoprevention.[29–31] Furthermore, these compounds could serve as synthetic probes useful for investigating the role of inositol phosphates in biochemical signaling pathways that cannot be tested using current methods. As a step toward these goals, the primary aim of this study was to develop an enzyme-stable $Ins(1,4,5)P_3$ analog. The structural basis for its design involved structure–activity relationships previously established for $Ins(1,4,5)P_3$ receptor binding and Ca^{2+} release activity (Fig. 1).[23,32–37] Much of the information known regarding receptor-binding specificity has been garnered from studies involving synthetic deoxy-analogs. Strategies for the syntheses of these compounds generally involve lengthy (i.e., of the order of 7–15 steps) modification of oxygenated carbohydrate precursors, including quebrachitol and D-galactopyranose.[38–40] The influence of deoxygenation at the 2- and 3-positions of the inositol framework was examined with 1D-3-deoxy and 1D-2,3-dideoxy-$Ins(1,4,5)P_3$,[39–41] which retain biological activities including receptor binding and Ca^{2+} mobilization. By contrast, analogs deoxygenated at the 6-position, such as 1D-2,3,6-trideoxy-$Ins(1,4,5)P_3$ and 1D-6-deoxy-$Ins(1,4,5)P_3$, displayed significantly lower-binding affinity to the $Ins(1,4,5)P_3$ receptor.[39,42]

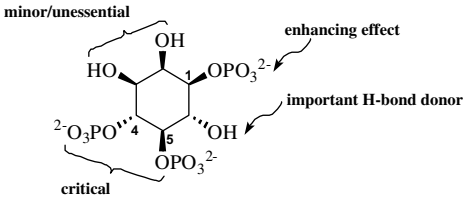


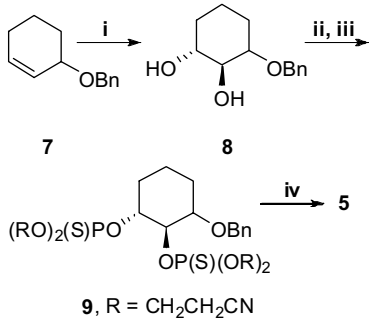
Figure 1. Previously established structure–activity relationships for $Ins(1,4,5)P_3$-receptor binding and calcium releasing.

Deoxygenation at specific positions of the inositol framework eliminates kinase phosphorylation sites,[39,43] and replacement of phosphate groups with phosphorothioates retains key interactions between the molecules and $Ins(1,4,5)P_3$ receptor, but imparts phosphatase resistance.[44–46] We report here the synthesis of two novel inositol phosphate analogs, **5** and **6**, which on the basis of these design principles are expected to be enzyme-stable receptor-binding analogs. An evaluation of their chemical and biochemical properties, including binding affinities for the $Ins(1,4,5)P_3$ receptor and resistance to enzymatic hydrolysis, is described.

2. Results

2.1. Chemistry

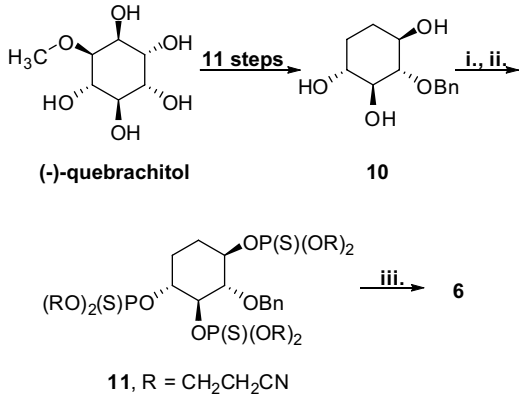
$Ins(4,5)P_2$ analog **5** is a deoxygenated analog bearing two phosphorothioate groups in the 4- and 5-positions and retaining the 6-hydroxyl group, which is critical for the binding of $Ins(1,4,5)P_3$ to its receptor (Fig. 1 and Chart 1).[36,37] While **5** is a putative $Ins(4,5)P_2$ analog, its synthesis was pursued primarily as a synthetic model and biochemical control substrate for investigating the $Ins(1,4,5)P_3$ analog **6**. Initially, we were interested in establishing chemistry needed to prepare and purify *trans*-vicinal phosphorothioates with a neighboring free alcohol. The synthesis of **5** is outlined in Scheme 1. Racemic diol **8** was prepared from cyclohexene-2-ol by the method of Savelli and co-workers.[47] First, cyclohexene-2-ol was protected as the benzyl ether **7** and subjected to *trans*-dihydroxylation using MCPBA (Scheme 1). The ^1H NMR analysis of the resulting white solid matched the published spectrum[48,49] and indicated that the resulting diol was obtained as a 1:1 mixture of diastereomers. A procedure for installation of the phosphorothioate groups was adapted from a phosphoramidite-based method reported by Potter and co-workers for the preparation of DL-*myo*-$Ins(1,4,5)P_3$.[46] Diol **8** was treated with excess bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite[50] and the resulting crude phosphate was allowed to react with sulfur to provide protected phosphorothioate ester **9**. Because **9** is the final compound bearing a chromo-



Scheme 1. Synthesis of analog **5**. Reagents: (i) MCPBA/H_2SO_4; (ii) tetrazole, bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite; (iii) sulfur, pyridine; (iv) Na, NH_3.

phore in the synthetic sequence, and considering difficulties encountered in the subsequent purification, we found it important to carefully purify the phosphorothioate ester by preparative reverse phase HPLC (UV detection). By this method the corresponding diastereomers could be resolved and separated if desired, but were routinely isolated as a mixture. Both the cyanoethyl and benzyl protecting groups were removed under basic reducing conditions (Na/NH_3), generating **5**. This material was isolated as the triethylammonium salt after a short DEAE-Sephadex anion exchange column, eluting with an increasing gradient of triethylammonium bicarbonate (TEAB), and visualized by Ellman's reagent.[51]

The synthesis of **6** was accomplished using analogous methods to those described above for the preparation of **5** from **10** (Scheme 2). Triol **10** was prepared in enantiomericaly pure form from (−)-quebrachitol using an 11-step linear sequence previously reported by Kozikowski et al.[39,41] Kozikowski's overall strategy for the preparation of **10** involves a series of selective protection steps in which selected pairs of vicinal hydroxyl groups are protected by exploiting their *cis*/*trans* and axial/equatorial relationships. Deoxygenation reactions are carried out on remaining free hydroxyl groups using Barton-type deoxygenation conditions. Each of two deoxygenation processes is preceded by a selective protection reaction and followed by extensive deprotection. While we found the reported strategy to be a reliable and reproducible method to obtain **10** on an approximately half-gram scale, drawbacks included its lengthiness, the need to separate an intermediate mixture of diastereomers (previous reports indicate that these intermediates are separable by column chromatography, but in our hands preparatory HPLC was required for satisfactory separation), and the stench/toxicity (thiol/tin) associated with deoxygenation reagents. Minor modifications that were made to published methods, including a procedure for HPLC separation of precursors to **10**, are indicated in the experimental section. Triol **10** was converted to the corresponding phosphates by treatment with bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite, followed by reaction with sulfur, and deprotection (Scheme 2). The final product was purified by anion exchange chromatography and analysis by HRMS displayed a molecular ion of 434.8963, consistent with the presence of three phosphorothioate moieties. Analysis by ^{31}P NMR displayed three resonances (46.58, 48.80, and 49.45), also consistent with the trisphosphorothioate structure. No ^{31}P resonances were observed in the 7.71–6.19 region,[39,41] which would be expected for phosphate groups, and no mass corresponding to phosphate (rather than phosphorothioate) groups were observed. The synthesis involved 13 steps and the separation of a 1:1 mixture of isomers; **6** was obtained in 4% overall yield.



Scheme 2. Synthesis of the analog **6**. Reagents: (i) tetrazole, bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite; (ii) sulfur, pyridine; (iii) Na, NH_3.

Analog **6** was purified and isolated as the triethylammonium salt using the same procedures employed in the preparation of **5**, as discussed above. The presence of **6** in chromatography fractions was determined using Ellman's reagent, which is used routinely as a sulfhydryl indicator (i.e., a visible yellow color is produced). Storage of aqueous **6** solutions at low temperature (−20 °C) for extended time (months) resulted in its partial decomposition, as indicated by MS analysis of aged samples in which a new peak with a negative ion *m*/*z* 433 was observed. This decomposition product could be removed by repurification on a DEAE-Sephadex anion exchange column. Furthermore, it was found that when treated with DL-dithiothreitol (DTT), a reagent useful for reducing disulfide bonds, the impurity with *m*/*z* 433 was completely re-converted to **6**. This relationship was confirmed by MS analysis as indicated in Figure 2. These data suggest that the impurity resulted from the formation of a disulfide bond between vicinal phosphorothioates. The corresponding equilibrium relationships and proposed structure **12** are illustrated in Scheme 3, and this material appeared to be stable to alkaline phosphatase-mediated hydrolysis (Fig. 4c).

2.2. Biochemistry

A competition-binding assay was carried out to determine the binding affinities of **5** and **6** with respect to the Ins(1,4,5)P_3 receptor. Equilibrium binding was determined relative to [^{3}H]Ins(1,4,5)P_3 using protein extract prepared from bovine adrenal cortex.[52] Ins(1,4,5)P_3, **5**, and **6** replaced receptor-bound [^{3}H]-Ins(1,4,5)P_3 in a concentration-dependent manner as

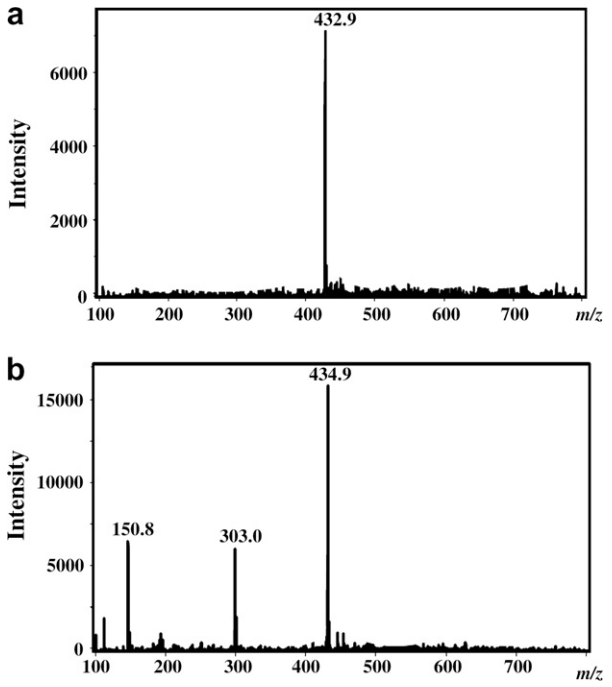


Figure 2. Full-scan MS of **12** after storage of **6** at −20 °C for a month (a, *m*/*z* 432.9); and full-scan MS of the same material after treatment with excess of DTT (b, *m*/*z* 434.9).

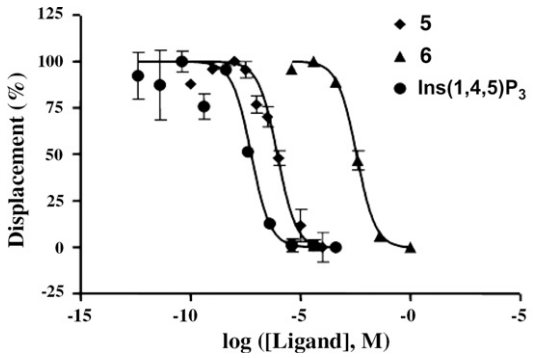
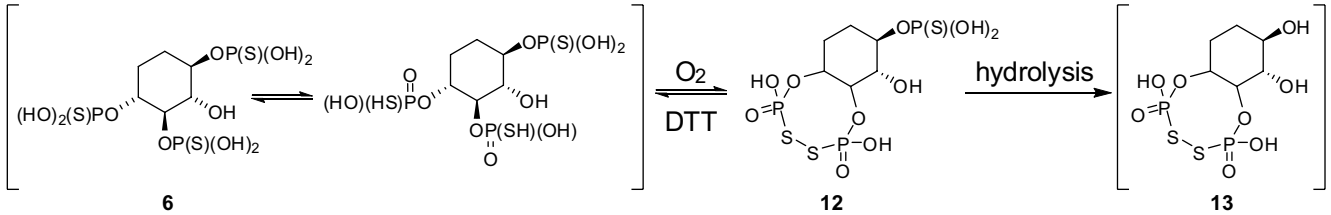


Figure 3. Effects of **5**, **6**, and Ins(1,4,5)P_3 on equilibrium competition binding of [^{3}H]Ins(1,4,5)P_3 to bovine adrenal cortical protein extracts.

illustrated in Figure 3. The strongest binding was demonstrated by Ins(1,4,5)P_3 (K_d 54 nM). The binding constant for **6** was in the high nanomolar range (K_d 810 nM), while binding of **5** was over three orders of magnitude higher. The displacing activities of adenosine 2′-monophosphate (AMP) and adenosine 5′-*O*-thiomonophosphate (AMPS) were also investigated to verify the specificity for Ins(1,4,5)P_3 and the analogs; neither demonstrated a measurable binding affinity for the receptor (data not shown).

The resistance of **6** to enzymatic degradation was addressed by reacting with alkaline phosphatase and analyzing the resulting mixture by LC/MS/MS using an electrospray ionization source (Fig. 4b). As a positive control, Ins(1,4,5)P_3 (*m*/*z* 419) was extensively hydrolyzed by alkaline phosphatase (30 min, 37 °C), producing *myo*-inositol-bisphosphate ($InsP_2$), *myo*-inositol-monophosphate ($InsP_1$), and inositol (Fig. 4a) with negatively charged [M−H]$^-$ ions *m*/*z* 339, 259, and 179, respectively. The identities of the inositol phosphates and inositol were verified by co-injection with authentic standards. To evaluate the stability of **6**, fragmentation data obtained for the products of Ins(1,4,5)P_3 incubation mixture were used to guide the anticipated product ions that would result from hydrolysis of **6**, for which authentic hydrolysis co-injection standards were not available. Thus, the products of the Ins(1,4,5)P_3 incubation mixture were selected for collision-induced dissociation (CID), and fragment ions for Ins(1,4,5)P_3 and $InsP_2$ of *m*/*z* 321 and 241, respectively, were observed (Fig. 4a). These fragment ions correspond to loss of orthophosphoric acid (H_3PO_4). The fragment ion generated from $InsP_1$ (*m*/*z* 179) corresponds to phosphate loss, and water loss for inositol (*m*/*z* 161). Data from the LC/MS/MS analysis of incubates of **6** with alkaline phosphatase under identical conditions as the positive control are shown in Figure 4b. The *m*/*z* values for the parent and fragment [M−H]$^-$ ions for **6** are 435 and 401, respectively. This fragmentation pattern is consistent with loss of SH_2. A product resulting from hydrolysis of **6** is expected to display *m*/*z* 339 and a fragment ion *m*/*z* 305, corresponding to loss of one phosphorothioate group. As indicated in Figure 4b, there is no evidence for the formation of this product under the phosphatase reaction conditions. As a further conformation of the validity of the selected fragment, evidence for the putative hydrolyzed product (i.e., observation of a new MS peak with *m*/*z* 339 that fragments to *m*/*z* 305) was observed by carrying out the same MS analysis of **6** after prolonged storage (several months). Furthermore, the same behavior (i.e., the formation of hydrolysis products) was observed for stored Ins(1,4,5)P_3. Finally, to determine whether **6** inhibits alkaline phosphatase, enzyme activity was monitored using a colorimetric assay with *p*-nitrophenyl phosphate (*p*NPP) as a substrate.[53] Phosphatase activity was determined at 37 °C by measuring the increase in absorbance at 405 nm that accompanies the hydrolysis of *p*NPP, and these data indicated



Scheme 3. Proposed decomposition pathway observed for **6** and proposed hydrolysis pathway. DTT, DL-dithiothreitol.

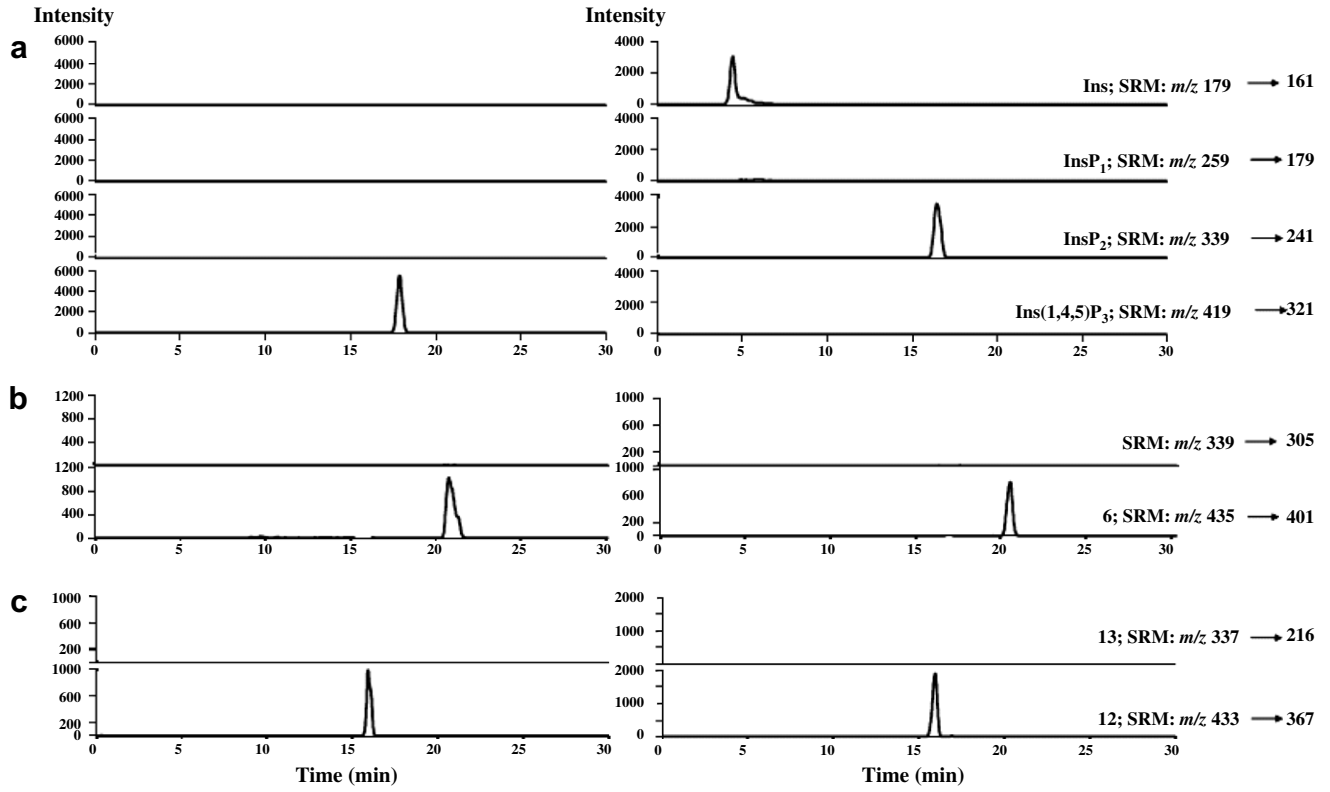


Figure 4. SRM chromatograms of reactions of $Ins(1,4,5)P_3$, **6**, or **12** with alkaline phosphatase. (a) $Ins(1,4,5)P_3$ control (left, unreacted $Ins(1,4,5)P_3$; right, $Ins(1,4,5)P_3$ hydrolyzed to $InsP_2$, $InsP_1$, and inositol), a small amount of $InsP_1$ is detected with rt 5.77 min. (b) **6** incubated with alkaline phosphatase. (Left, unreacted **6**; right, sample after incubation indicates that starting compound remains intact, and there is no evidence for a hydrolysis product.) (c) **12** incubated with alkaline phosphatase. (Left, unreacted **12**; right, sample after incubation indicates that starting compound remains intact, and there is no evidence for **13**.) Monitored SRM transitions are indicated on the right for each channel.

that **6** does not inhibit alkaline phosphatase activity at high micromolar range (data not shown).

3. Discussion

The design of the inositol phosphate analogs described in this study relied on known structure–activity relationships for $Ins(1,4,5)P_3$ (Fig. 1), indicating that vicinal 4,5-phosphate groups are required for $Ins(1,4,5)P_3$ Ca^{2+}-releasing activity, and that the 1-phosphate acts as an enhancer.[33,54] Replacing $Ins(1,4,5)P_3$'s phosphate groups by phosphorothioate groups has been found to provide resistance to phosphatase-catalyzed hydrolysis without greatly affecting binding properties,[46,55] but the analog used in the study may still be phosphorylated at the 2- and 3-positions. Therefore, deoxygenation at these positions was a strategy utilized to block phosphorylation. 1D-2,3-dideoxy-$Ins(1,4,5)P_3$ maintains a relatively high affinity for the $Ins(1,4,5)P_3$ receptor and is a full agonist, releasing Ca^{2+} from permeabilized SH-SY5Y cells.[41] In human cells, phosphorylation of $Ins(1,4,5)P_3$ has been observed at the 3-position,[56] and the substrate of the known 6-kinase is not $Ins(1,4,5)P_3$, but $Ins(1,3,4)P_3$, which is formed by the hydrolysis of $Ins(1,3,4,5)P_4$.[57] In addition, 1D-6-deoxy-*myo*-$Ins(1,4,5)P_3$ was a full agonist, but is 70-fold less potent than $Ins(1,4,5)P_3$, indicating that the 6-hydroxyl group is important for receptor-binding and Ca^{2+} release,[37,42] and it is therefore present in **6**.

Inositol phosphate multikinases catalyze phosphorylation of the 3- and 6-hydroxyl groups of $Ins(1,4,5)P_3$, but in cells 3-phosphorylation is the dominant product.[58] Although known enzyme profiles indicate that **6** is expected to be kinase-stable, further studies are required to explicitly address the susceptibility toward phosphorylation by specific kinases. As a preliminary test of enzyme stability carried out in this study, we used alkaline phosphatase, which efficiently hydrolyzes $Ins(1,4,5)P_3$. In cells, $Ins(1,4,5)P_3$ 5-phosphatase is the specific enzyme that hydrolyzes $Ins(1,4,5)P_3$, but it has low substrate-specificity compared with $Ins(1,4,5)P_3$ 3-kinase.[59] Many synthetic inositol phosphates are high-K_i $Ins(1,4,5)P_3$ 5-phosphatase inhibitors,[39,41,55,59] such as *myo*-inositol 1,4,5-trisphosphorothioate, suggesting that **6** is a potential $Ins(1,4,5)P_3$ 5-phosphatase inhibitor. The current studies indicate that **6** is stable to general enzyme-mediated hydrolysis, but further studies with specific kinases and phosphatases and in the complex environment of the cell are required to determine the biological stability of **6**.

Thiophosphate **6** has a significant binding affinity (810 nM) for the $Ins(1,4,5)P_3$-receptor despite its struc-

tural modification. However, the presence of only the 4,5-phosphorothioates in **5** results in a dramatic decrease in binding affinity, consistent with the specificity of the ligand–receptor interaction. The X-ray structure of the Ins(1,4,5)P_3-binding domain of mouse type-I Ins(1,4,5)P_3 receptor with bound Ins(1,4,5)P_3 indicates interactions of specific Arg and Lys residues with the three phosphate groups,[60] and replacement by phosphorothioates therefore is expected to influence the interaction. Compared to other phosphatase-stable Ins(1,4,5)P_3 analogs, including 1L-(+)-*myo*-Ins(1,4,5)P_3,[55] 1D-6-deoxy-Ins(1,4,5)P_3S_3, and L-ch-Ins(2,3,5)P_3S_3,[61] **6** retains a notably high affinity for the Ins(1,4,5)P_3 receptor. Three closely related Ins(1,4,5)P_3-receptor subtypes have been characterized and they are differentially expressed and involved in physiological functions such as regenerating Ca^{2+} signals.[16] The previously reported analog 2-deoxy-1,4,5-Ins(1,4,5)P_3 had a slightly greater affinity for type 2 and type 3 receptors, whereas 3-deoxy-1,4,5-Ins(1,4,5)P_3 showed greater selectivity for type 3 receptor.[62] To define specific ligand–receptor-binding interactions for **6**, further studies are needed to determine the selectivity of **6** for different Ins(1,4,5)P_3 receptors.

Techniques to assess the stabilities of inositol phosphates and analogs have included NMR, that is, monitoring ^{31}P signals for enzymatic reactions performed in an NMR tube,[63] radioflow HPLC after incubation with [^{3}H]Ins(1,4,5)P_3,[37] or using a functional assay (i.e., measuring Ca^{2+} mobilization).[22] The requirement for large amounts of material and low sensitivity and accuracy are drawbacks for NMR analysis, and preparations of isotope-labeled analogs and tedious sample preparation are generally required for the radioflow methods. Finally, functional assays do not necessarily transfer for the analysis of structural analogs. To overcome limitations in these methods, we developed a new LC/MS/MS analytical method for inositol phosphate analog-enzyme incubations. This method is sensitive and fast, and sample preparation is easy. This method was used here to investigate analog stability toward phosphatase activity, but it has potential applications for further studies of inositol phosphate kinase activity and analysis of complex inositol phosphate mixtures.[64]

4. Conclusion

In conclusion, we have synthesized **5** and **6**, two novel deoxygenated phosphorothioate analogs of the inositol phosphates Ins(1,4,5)P_3 and Ins(4,5)P_2. Phosphorothioate **6** is stable to enzymatic hydrolysis, but a tendency of the vicinal phosphorothioates to form disulfide bonds was observed indicating limitations in its chemical stability. This analog specifically binds to Ins(1,4,5)P_3 receptor with a K_d of 810 nM, which compares favorably with previously reported Ins(1,4,5)P_3 phosphorothioate analogs.[55,61] Future studies will be aimed at characterizing cellular responses to **6** and using it to probe the role of Ins(1,4,5)P_3 and its receptor in physiological processes.

5. Experimental

5.1. General details

THF was obtained anhydrous in sure-seal bottles from Aldrich and used without further purification. Flash chromatography was performed using 70–230 mesh Silica gel purchased from Aldrich Chemical Co. (Milwaukee, WI). Silica gel 60 mesh TLC plates were obtained from Merck (Lawrence, KS). Tetrazole was obtained from Glen Research (Sterling, VI) as a saturated solution in anhydrous acetonitrile and used as provided. Ammonia was used from a lecture bottle, 99.9% pure, obtained from Aldrich. DEAE-Sephadex A-25 anion exchange resin was obtained from Sigma Chemical Co. (St. Louis, MO) and was packed in a glass column with water. Alkaline phosphatase was supplied from Sigma in a 50% glycerol solution with 5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$; activity was assessed at 4900 U/mg protein with 11.2 mg protein/mL. Amicon Centricon YM-3 centrifuge filters were obtained from Millipore and were rinsed with 2 mL H_2O for 3 h at 7000*g* prior to use. [^{3}H]Ins(1,4,5)P_3 was obtained from American Radiolabeled Chemicals (St. Louis, MO) and used without further purification. Radioactivity counting was done using Eco Scint liquid scintillant from National Diagnostics (Atlanta, GA). The phosphoramidite coupling reagent, bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite, was prepared as previously described[50] and the ^{1}H and ^{31}P NMR of the material obtained matched the published spectra.[65] 1D-6-*O*-Benzyl-1,2,3-trideoxy-*myo*-inositol **8** was prepared by the method of Savelli and coworkers[47] and the ^{1}H NMR matched the published spectrum.[48,49] Ellman's reagent was prepared and used to detect phosphorothioate compounds in fractions after anion exchange chromatography as described previously.[51]

Preparative HPLC was carried out in all systems using two Waters Associates pumps (Waters Division, Millipore, Milford, MA), with a UV detector at 254 nm (Shimadzu model SPD-10A). System 1: at a flow rate of 5 mL/min, a Phenomenex Luna 5 μm C_{18} 250 × 21.2 mm column was eluted isocratically for 5 min with 5% CH_3OH/95% aqueous 40 mM NH_4OAc, then with a gradient from 5% to 100% CH_3OH over a course of 20 min. An isocratic flow of 100% CH_3OH was then maintained. System 2: at a flow rate of 5 mL/min, a Phenomenex Luna 5 μm C_{18} 250 × 21.2 mm column was eluted isocratically with 9:1 MeOH/H_2O.

Direct injection mass spectrometry analysis was performed for compound characterization on an Agilent 1100 LC/MSD ion trap instrument (Agilent Technologies, Inc., Wilmington, DE) operating in negative-ion mode.

5.2. Synthesis of compounds 5–11

5.2.1. Benzyl 2-cyclohexen-1-yl ether (7). 2-Cyclohexen-1-ol (2 mL, 20 mmol) was added to a slurry of NaH (1 g, 42 mmol) in THF at 25 °C under an atmosphere of nitrogen. After 1 h, benzyl chloride (6 mL, 52 mmol)

was added and the mixture was stirred for 12 h. Excess NaH was quenched by addition of MeOH. Solvents were removed by rotary evaporation and the resulting residue was dissolved in diethyl ether (80 mL). This organic solution was transferred to a separatory funnel, extracted three times with water, and dried over Na_2SO_4. The solution was filtered and solvents removed by rotary evaporation. The residue was concentrated under high vacuum to remove any methyl benzyl ether and then purified by column chromatography (hexanes, followed by Ethyl Acetate/hexanes 1:9 v/v) to provide 1.7 g (45% yield) of the title compound as a colorless oil. ^{1}H NMR matched the published spectrum.[66]

5.2.2. 1D-6-*O*-Benzyl-1,2,3-trideoxy-*myo*-inositol (8). To a stirred dispersion of **7** (0.9 g, 5 mmol) in water (30 mL) was added MCPBA in small portions over a period of 5–10 min at 0 °C. The mixture was warmed to 25 °C and stirred for 4 h. To the mixture then was added H_2SO_4 (1 mL of a 10% solution) and stirring was continued for 12 h. NaOH (1 M solution) was added to the reaction mixture to adjust the pH to 7. This aqueous solution was saturated with NaCl and extracted three times with ethyl acetate. The combined organic fractions were dried over Na_2SO_4, filtered, and concentrated. Purification by flash chromatography (ethyl acetate) afforded the title compound as a white solid (330 mg, 31% yield). ^{1}H NMR matched the published spectrum.[48,49]

5.2.3. 1D-1,2,3-Trideoxy-*myo*-inositol 4,5-bisphosphorothioate (5). 1D-6-*O*-Benzyl-1,2,3-trideoxy-*myo*-inositol **8** (330 mg, 1.5 mmol) was dissolved in an acetonitrile solution of tetrazole (40 mL, 18 mmol) under a N_2 atmosphere. Bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite[50] (2.3 g, 8.48 mmol) was added. The reaction mixture was stirred at 23 °C under N_2 for 2 h, and turned cloudy. Solvent was removed under vacuum and the resulting residue was re-dissolved in pyridine (10 mL). Sulfur (460 mg, 14.3 mmol) was added and the reaction mixture was stirred for 24 h. Solvent was evaporated and crude product was dissolved in MeOH (~3 mL) and filtered through a nylon disk. The solution was concentrated and purified by flash column chromatography with a gradient from 25:75 EtOAc/hexanes to 50:50 EtOAc/hexanes. Subsequent purification by HPLC from multiple injections using System 1 yielded 299 mg **9** (0.48 mmol, 32%). Retention time was at 47 and 52 min (~1:3 mixture of diastereomers). This material was dissolved in dry dioxane (6 mL) and a portion (2 mL) of this solution was added to liquid ammonia, of which approximately 50 mL had been condensed in a three-necked flask fitted with a dry ice/acetone-filled condenser. To this mixture was added Na (290 mg, 13 mmol) in small pieces producing a deep blue solution. The mixture was stirred at 25 °C for 10 min. The reaction mixture was quenched by adding ethanol (4 mL). NH_3 was removed under a flow of N_2 over the course of ~2 h. The remaining solution was concentrated under vacuum and crude residue was purified by anion exchange chromatography on DEAE-Sephadex A-25 resin with a stepwise gradient from water to 0.25 M triethylammonium bicarbonate (TEAB), 0.5 mM TEAB, 0.75 mM TEAB, finishing with 1 M TEAB. The presence of phosphorothioate was detected by Ellman's reagent in column fractions. Solvent was removed and the title compound isolated as its triethylammonium salt (91 mg, 0.11 mmol, 7.2% yield). ^{1}H NMR (300 MHz, D_2O, mixture of diastereomers) δ 1.35–1.60 (m, 4H), 1.70–1.80 (m, 1H); 1.95–2.05 (m, 1H); 3.40–3.50 (m, 1H); 3.85–4.00 (m, 2H); 4.15 (m, 0.5H); 4.35 (m, 0.5H). ^{31}P NMR (300 MHz, D_2O) δ 48.3 (d, J = 146 Hz), 46.7 (d, J = 230 Hz); HRMS calculated for $[C_6H_{13}O_7P_2S_2]$ 323.2407. Found: 322.9584.

5.2.4. 1D-6-*O*-Benzyl-2,3-dideoxy-*myo*-inositol (10). The title compound was prepared by the method of Kozikowski et al. with minor modifications as follows.[39] Isomers of 1D-4,5-di-*O*-benzoyl-6-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidine-*myo*-inositol were separated by preparatory HPLC, using System 2, and 1D-4,5-di-*O*-benzoyl-6-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidene-*myo*-inositol (35–36 min) eluted after 1D-5,6-di-*O*-benzoyl-4-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidene-*myo*-inositol (29.5–30.5 min). 1D-4,5-Di-*O*-benzoyl-6-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidine-*myo*-inositol (1.13 g, 2.25 mmol) was dissolved in methanol (50 mL) and *p*-toluenesulfonic acid (140 mg, 0.736 mmol) was added. The reaction mixture was allowed to stir at 23 °C. After 3.5 h, water (approx. 5 mL) was added to the reaction mixture, and the resulting solution was extracted with CH_2Cl_2 (3× 20 mL). The combined organic solutions were washed sequentially with saturated sodium bicarbonate and brine, dried over Na_2SO_4, and solvent was evaporated to yield 0.53 g of **10** (2.21 mmol, 98%) as a white solid. ^{1}H NMR matched published data.[39]

5.2.5. 1D-6-*O*-Benzyl-2,3-dideoxy-*myo*-inositol 1,4,5-tris[di-(2-cyanoethyl)phosphorothioate] (11). 1D-6-*O*-Benzyl-2,3-dideoxy-*myo*-inositol **10** (52 mg, 0.218 mmol) was dissolved in an acetonitrile solution of tetrazole (8.5 mL, 3.825 mmol) under a N_2 atmosphere. A solution of bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite[50] (575 mg, 2.12 mmol) in CH_2Cl_2 (2.5 mL) was added dropwise. The reaction mixture was stirred at 23 °C under N_2 for 2.5 h. Solvent was removed under vacuum and the resulting residue was re-dissolved in pyridine (2.5 mL). Sulfur (107 mg, 3.34 mmol) was added and the reaction mixture was stirred for 24 h. Solvent was evaporated and crude product was purified by flash column chromatography with a gradient from 20:80 EtOAc/hexanes to EtOAc. Subsequent purification by HPLC using System 1 yielded 108 mg **11** (rt 28.5 min) (0.128 mmol, 57%). TLC R_f 0.33 (2:1 ethyl acetate/hexanes). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26–7.43 (m, 5H), 4.85 (s, 2H), 4.66 (m, 1H), 4.50 (m, 2H), 4.29–4.40 (m, 8H), 4.19 (m, 2H), 4.10 (m, 2H), 3.85 (m, 2H), 3.65 (dd, J = 8.7 Hz, 1H), 2.71 (m, 8H), 2.22–2.44 (m, 4H), 2.15 (m, 1H), 1.65 (m, 2H), 1.28 (m, 3H), 0.85 (m, 1H); ^{31}P NMR (300 MHz, CDCl3) δ 66.26, 67.13, 67.96; HRMS calculated for $[C_{31}H_{39}N_6O_{10}P_3S_3]$ 844.1103. Found: 844.1113.

5.2.6. 1D-2,3-Dideoxy-*myo*-inositol 1,4,5-trisphosphorothioate (6). A 250 mL three-necked round bottom flask fitted with a condenser was flushed with N_2 and cooled

to −78 °C. Ammonia (50 mL) was condensed into the flask at −78 °C. A solution of 1-D-6-*O*-benzyl-2,3-dideoxy-*myo*-inositol 1,4,5-tris[di-(2-cyanoethyl)phosphorothioate] **11** (21 g, 25 mmol) in anhydrous dioxane (3 mL) was added dropwise while the solution was stirred. Na, stored in mineral oil, was slowly cut into small pieces, rinsed with Et_2O, and immediately added to the reaction mixture. After 1–2 min, a color change to dark blue was observed and this solution was stirred at −78 °C for 10 min. The reaction was quenched by dropwise addition of MeOH (approx. 3 mL) and solvent was evaporated by warming to 23 °C under a N_2 purge. Crude residue was purified by anion exchange chromatography on DEAE-Sephadex A-25 resin with a stepwise gradient starting with 0.3 mM triethylammonium bicarbonate (TEAB) to 0.7 mM TEAB. The presence of phosphorothioate in column fractions was detected by Ellman's reagent in column fractions, yielding 5.45 mg **6** isolated (32%) as a yellowish white solid. ^{1}H NMR (D_2O) δ 4.21 (m, 2H), 3.63 (m, 1H), 2.21 (m, 2H), 1.47 (dd, $J = 9.0$, 2H); ^{31}P NMR (D_2O) δ 46.6, 48.8, 49.4; HRMS calculated for [C_6 H_{14} O_{10} P_3 S_3]: 434.8962. Found: 434.8963.

5.3. Binding assay

Bovine adrenal cortical protein extract was prepared as previously described.[52] Protein concentration was determined to be 17.3 mg/mL by Bradford Assay.[67] The binding assay was performed using the method of Challiss et al.[52] Samples contained protein solution (30 μL, 0.519 mg), Tris/HCl/EDTA buffer (30 μL, 100 mM Tris/4 mM EDTA, pH 8.0), [^{3}H]Ins(1,4,5)P_3 (30 μL aqueous solution, approx. 7000 dpm), and 30 μL aqueous solution of analyte. Samples were vortex-mixed every 5 min through a 45-min incubation period at 4 °C. The incubation mixtures then were filtered over Whatman GF/B filters and washed 4× 2.5 mL with ice cold buffer (25 mM Tris/HCl/5 mM $NaHCO_3$/1 mM EDTA, pH 8.0). Non-specific binding was determined using a receptor-saturating concentration of **6** (100 mM). The amount of radioactivity bound to the receptor was counted by soaking the filter papers in liquid scintillant for 36 h.

5.4. Reaction of 6 with alkaline phosphatase and HPLC/MS/MS analysis

In a volume of 300 μL, **6** (200 μmol) or Ins(1,4,5)P_3 (80 μmol) was incubated with alkaline phosphatase (6 μL, activity 62 U/μL) for 30 min at 37 °C. The resulting mixtures were first filtered by micro YW3000 (Micron Technology, Inc. CA) at 4 °C, then centrifuged at 7000 rpm for 30 min to remove the enzyme. Four microliters of the filtered solution per injection was introduced with an autosampler into the ESI source using a Biobasic AX 150 × 0.5 mm weak anion exchange capillary column. The flow rate was 10 μL/min, solvent A 95:5 mixture of water/methanol, solvent B 200 mM solution of $(NH_4)_2CO_3$. Initial solvent conditions (100% A) were changed with a linear gradient over the first 3 min to 50:50 B/A, then to 72:28 B/A over a course of 25 min, then returning to 100% A in 2 min. The retention times and molecular weights observed for compounds of interest are as follows: Ins(1,4,5)P_3, 17.91 min, $[M-1]^-$ 419, Inositol, 4.44 min, $[M-1]^-$ 179; InsP_1, 5.77 min, $[M-1]^-$ 259; InsP_2, 16.42 min, $[M-1]^-$ 339; **6**, 20.36 min, $[M-1]^-$ 435; hydrolyzed **6**, 16.79 min, $[M-1]^-$ 339; **12**, 15.93 min, $[M-1]^-$ 433; **13**, 6.34 min, $[M-1]^-$ 337. Negative ESI-MS/MS was performed using the following parameters: spray voltage 5.0 kV; sheath gas pressure, 31; capillary temperature, 330 °C; collision energy, 19 V; scan width, 0.3 amu; scan time, 0.25 s; Q1 peak width, 0.7 amu; Q3 peak width, 0.7 amu; Q2 gas pressure, 1.5 mTorr; source CID, −16 V; and tube lens offset, 59–69 V; MS/MS data were acquired and processed by Xcaliber software version 1.4 (Thermo Electron).

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