STUDIES ON BIOSYNTHESIS OF THE LINKAGE REGION OF CHONDROITIN SULFATE-PROTEIN COMPLEX*

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In the native state, chondroitin-4-sulfate is covalently bound to protein. Since the original studies of Muir,1 which implicated serine in this linkage, Rodén et al. in this laboratory have demonstrated that the linkage region between protein and polysaccharide in heparin,2 chondroitin-4-sulfate,3, 4 and dermatan sulfate5 has the following structure: GlcUA-Gal-Gal-Xyl-serine. Other studies6, 7 have indicated that mucopolysaccharides of a wide variety of tissues of different species contain a similar linkage region.

Previous publications have shown that a cell-free particulate enzyme derived from embryonic chick cartilage catalyzes the synthesis of chondroitin sulfate from the appropriate uridine nucleotide sugars.8—10 Since the CS isolated from embryonic chick cartilage was found to contain serine, xylose, and galactose with molar ratios of 1.00:1.32:2.06,11 it seemed logical to use the same enzyme preparation for studies on the biosynthesis of the linkage region. The transfer of xylose-C14 from UDP-Xyl-C14 to protein has been previously reported from this laboratory.12

Bdolah and Feingold13 have found that UDP-GlcUA can be converted to UDP-Xyl by a crude enzyme preparation from hen oviduct. Grebner et al.14, 15 reported the transfer of xylose from UDP-Xyl to protein using cell-free preparations derived from hen oviduct and mouse mastocytoma. The linkage of xylose to serine in the protein was demonstrated.

The incorporation of galactose from UDP-Gal into glycoproteins, catalyzed by cell-free preparations, has been reported by a number of laboratories.16—18

This paper presents evidence for the transfer of both xylose and galactose from the corresponding uridine diphosphate sugars to protein by a cell-free preparation from embryonic chick cartilage.

Materials and Methods.—UDP-GlcUA-U-C14 (125 mc/mmole) and UDP-Gal-U-C14 (120 mc/mmole) were purchased from the New England Nuclear Corp. UDP-GlcUA, UDP-Xyl, UMP-morpholidate, and pronase were purchased from the California Biochemical Corp. UDP-Gal was prepared as described by Roseman et al.19 UDP-Xyl-U-C14 (104 mc/mmole) was kindly prepared by Drs. H. Ankel and D. S. Feingold from UDP-GlcUA-C14 with purified UDP-glucuronate carboxy-lyase from Cryptococcus laurentii.20 O-β-D-xylosyl-L-serine, 4-O-β-D-galactosyl-D-xylose, and β-galactosidase were gifts from Dr. L. Rodén.

Paper chromatography was performed on Whatman no. 1 or no. 3 MM papers in the following solvents: (A) isobutyric acid: 2 N ammonia (5:3); (B) n-butanol:pyridine:acetic acid:water (15:10:3:12); (C) phenol:water:8-hydroxyquinoline (8:2:0.01 by weight); (D) t-pentanol:isopropanol:water (8:2:3); (E) 1.5 M sodium phosphate pH 6.0; (F) ethyl acetate:pyridine:water (8:2:1); (G) n-butanol:acetic acid:water (12:3:5); (H) n-butanol:pyridine:water (1:1:1).

Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer (model 314 EX) by methods previously described.9

Preparation of enzyme: Epiphyses of femurs and tibias from fifty to fifty-five 13-day-old chick embryos were dissected free of soft tissues, chilled, and homogenized in 12 ml of buffer solution. Initially, a buffer of the following composition was used: Tris, 0.05 M (pH 7.6); KCl, 0.07 M; MgCl2, 0.03 M; EDTA, Na2, 0.001 M, cysteine-HCl, 0.006 M (buffer A). In later experiments,
the tissue was homogenized in 0.2 M sucrose, 0.001 M EDTA, 0.006 M cysteine-HCl adjusted to pH 7.0 with KOH (buffer B).

The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant fraction was used. In some experiments the 10,000 g supernatant solution was further fractionated by centrifugation at 105,000 g for 90 min. The 105,000 g supernatant solution was used directly and the 105,000 g pellet after resuspension in buffer by gentle hand homogenization.

**Isolation of labeled protein:** After addition of an equal volume of 10% TCA, the incubation mixtures were chilled rapidly in an ice bath and kept at 0-4° for 30 min. Precipitates were collected by centrifugation and washed five times with 5-ml portions of 5% TCA, twice with 50% ethanol:ether (v/v), and once with ether. After the precipitates were dried, they were dissolved in a small volume of 1.0 N KOH and diluted to an alkali concentration of 0.1 N or less. Samples were assayed for radioactivity, and protein was determined by the Biuret method using bovine serum albumin as a standard.

**Formation of DNP-xylosylserine:** A mixture of xylosylserine (0.5 µmole), 20 µl of fluorodinitrobenzene, 20 µl of triethylamine, and 1.0 ml of water was shaken for 6 hr in the dark, acidified to pH 2, and extracted five times with 5-ml portions of ether. The DNP-xylosylserine was extracted from the aqueous residue with ethyl acetate. Ethyl acetate was removed under vacuum, and the residue was dissolved in a small volume of water.

**Desalting on ion exchange resin:** Solutions of neutral sugars were desalted by passage over a column of Dowex 50-X8 (H+ form, 100-200 mesh, 1 × 20 cm) and a column of Dowex 1-X8 (acetate form, 100-200 mesh, 1 × 20 cm).

**Isolation of the products of alkaline degradation of the C14-labeled TCA precipitates:** Galactose-C14 and xylose-C14-labeled TCA precipitates were separately dissolved at room temperature in 2.5 ml of 0.5 N KOH containing 15 mg/ml of KBH4. After 2 hr at room temperature, 3 vol of 10% TCA were added to neutralize the solutions and precipitate the protein. The supernatant solutions were passed over Dowex 50-X8 (H+, 100-200 mesh, 1 × 20 cm) and extracted five times with equal volumes of ether to remove the TCA. Boric acid was repeated to remove the concentration to dryness in a rotary evaporator following addition of methanol each time. This step was found to be critical: attempts to desalt the sugar alcohol on Dowex 1 resin without prior removal of the borate resulted in poor recoveries. The residues were dissolved in water, desalted, and the solutions were evaporated to dryness, dissolved in 0.25 ml of water, and chromatographed in solvent D.

**Digestion with β-galactosidase:** Samples were digested at room temperature for 20 hr with 15 µg of crystalline β-galactosidase in 0.03 ml of Tris-acetate buffer (pH 7.5). At the end of the digestion, the entire solution was chromatographed on Whatman 3 MM paper in solvent D.

**Results.—Incorporation of xylose-C14:** The results in Table 1 show that the incorporation of xylose-C14 into TCA-precipitable protein was catalyzed by the 10,000 g supernatant fraction of chick embryonic cartilage. Further fractionation of this preparation results in an approximately equal partition of the activity between the 105,000 g supernatant fraction and the 105,000 g pellet fraction. The

| TABLE 1  
| INCORPORATION OF XYLOSE-C14 INTO TCA-PRECIPITABLE PROTEIN |
|-----------------|-----------------|-----------------|-----------------|
| Enzyme fraction | Substrates for protein synthesis | Substrates for CS synthesis | Incorporation (total cpm) |
| 10,000-g Supernatant fraction (boiled control) | + | + | 0 |
| 10,000-g Supernatant fraction | + | + | 14,000 |
| 10,000-g Supernatant fraction | - | - | 19,400 |
| 105,000-g Pellet fraction | + | + | 6,300 |
| 105,000-g Supernatant fraction | + | + | 5,200 |

Epiphyses from 50 embryo chicks were homogenized in 15 ml of buffer A. Each tube contained 1.0 ml of the appropriate enzyme preparation, ATP (2.0 µmole), UDP-Xyl-C14 (6.75 µmoles, 7 × 10⁶ cpm), and the additions in a final volume of 1.3 ml. After 60 min at 37° the reactions were stopped by immersion of the tubes in a boiling water bath for 3 min, the protein was precipitated with TCA and assayed for radioactivity as outlined in Methods.

* UDP-GlcUA, 0.6 µmole; UDP-GalNAc, 0.24 µmole; UDP-Gal, 0.1 µmole. 
** UDP-GLC, 0.1 µmole; 20 amino acids, 0.2 µmole; PEP, 1.0 mg; PEP-kinase, 200 µg.**
incorporation of xylose-C\textsuperscript{14} was not stimulated by the presence of substrates necessary for the cell-free synthesis of protein or chondroitin. This would imply that the xylose-C\textsuperscript{14} is transferred to a protein acceptor already present in the homogenate. In all subsequent experiments the 10,000 g supernatant fraction was used.

The incorporation of xylose was linear with time over a period of 40 minutes at 37°. Figure 1 shows the dependence of xylose-C\textsuperscript{14} incorporation on pH; the optimum incorporation occurs at pH 6.5 in 0.05 M Tris-acetate buffer. Under the conditions tested, addition of manganous or magnesium ions stimulated incorporation (Fig. 2).

**Characterization of the xylose-C\textsuperscript{14} product:** A sample of the xylose-C\textsuperscript{14}-labeled TCA precipitate (15,000 cpm) was hydrolyzed in 1.0 N HCl for 15 minutes at 100°. The hydrolysate was desalted as described in Methods; the eluate (9,480 cpm, 63%) was analyzed by paper chromatography in solvent F. All the radioactivity moved to the same position as a xylose standard.

**Alkaline degradation of the xylose-C\textsuperscript{14}-labeled protein:** O-substituted serine or threonine derivatives readily undergo \(\beta\)-elimination under mild alkaline conditions. This reaction requires that the carboxyl group of the serine or threonine must be bound in ester or amide linkage.\textsuperscript{22} Figure 3 shows that mild alkali treatment of the xylose-C\textsuperscript{14}-labeled protein results in solubilization of more than 90 per cent of the original radioactivity.

The radioactive xylose was recovered as xylitol-C\textsuperscript{14} when xylose-labeled protein (27,800 cpm) was treated with alkali and borohydride as outlined in Methods. After

\[\text{Fig. 1.—Epiphyses from 51 chick embryos were homogenized in 20 ml of buffer B containing 0.012 M MgCl}_2. \text{ Each tube contained 0.9 ml of the 10,000 g supernatant solution, Tris-acetate buffer of the desired pH (50 \(\mu\) moles of Tris), and UDP-Xyl-C\textsuperscript{14} (0.14 \(\mu\) mole, 0.14 \(\mu\) c) or UDP-Gal-C\textsuperscript{14} (0.010 \(\mu\) mole, 0.17 \(\mu\) c) in a final volume of 1.0 ml. After 50 min at 37° the reactions were stopped by the addition of TCA, and the precipitates were isolated and assayed for radioactivity as outlined in Methods. The solid line shows the incorporation of xylose-C\textsuperscript{14}, and the broken line that of galactose-C\textsuperscript{14}.} \]

\[\text{Fig. 2.—Epiphyses from 45 chick embryos were homogenized in 15 ml of buffer B. The xylose incubations contained 0.9 ml of the 10,000 g supernatant preparation (total volume, 11.0 ml), Tris-acetate buffer pH 6.5 (50 \(\mu\) moles of Tris), and UDP-Xyl-C\textsuperscript{14} (0.064 \(\mu\) mole, 0.139 \(\mu\) c) in a final volume of 1.0 ml. Epiphyses from another 44 embryo chicks were homogenized in 20 ml of buffer B. The galactose incubations contained 0.9 ml of the 10,000 g supernatant preparation (18.0 ml), Tris-acetate buffer pH 5.5 (50 \(\mu\) moles of Tris), and UDP-Gal-C\textsuperscript{14} (0.0105 \(\mu\) mole, 0.18 \(\mu\) c) in a final volume of 1.0 ml. MgCl\textsubscript{2} and MnCl\textsubscript{2} were added as shown in the figure. After 40 min at 37° the reactions were stopped by addition of TCA, and the precipitates were isolated and assayed for radioactivity as outlined in Methods. ▲, Galactose incorporation with added MnCl\textsubscript{2}; \(\Delta\), galactose incorporation with added MgCl\textsubscript{2}; ○, xylose incorporation with added MnCl\textsubscript{2}; and ◆, xylose incorporation with added MgCl\textsubscript{2}.} \]
removal of salt, the recovered radioactive material (27,000 cpm, 97%) was analyzed by paper chromatography in solvent D. Figure 4 shows that the radioactivity migrated to the same position as xyitol standard.

Isolation of xylosyl-\(^{14}\)C-serine: Xylose-labeled protein was prepared with 15 ml of supernatant enzyme derived from 50 embryo chicks. The enzyme was incubated in buffer A with UDP-Xyl-\(^{14}\)C (24 \(\mu\)moles; \(2.8 \times 10^6\) cpm) for 60 minutes at 37°C. The labeled protein was digested with 10 mg of pronase at 37°C in 5 ml of Tris-acetate buffer (0.1 M in Tris, pH 8.0) containing 0.001 M CaCl\(_2\) and 7 per cent ethanol. After 48 hours an additional 15 mg of pronase was added and the digestion was continued for 24 hours. The incubation mixture was heated for three minutes in a boiling water bath, and the protein was removed by centrifugation. All the radioactivity (1.2 \(\times\) 10\(^6\) cpm; 1.03 \(\mu\)moles of xylose) was solubilized by the pronase, and the pellet was discarded. A sample of the supernatant solution (90,000 cpm) was applied to a column of Dowex 50-X8 (\(H^+\), 100–200 mesh, 1 \(\times\) 20 cm). About 30 per cent of the radioactivity (28,000 cpm) was eluted with a water wash, and 51 per cent (46,000 cpm) was eluted with 2.0 \(M\) pyridine.

The pyridine eluate was concentrated to dryness several times, dissolved in a small volume of water, and chromatographed in solvents A, B, and C. Approximately 40 per cent of the radioactivity moved to the same position as authentic xylosylserine in each solvent, but several other radioactive peaks, presumably small peptides, and a number of ninhydrin-positive spots were observed. The radioactive xylosylserine was further purified and characterized by preparative paper chromatography on Whatman 3 MM paper in solvent H. The area corresponding to xylosylserine was eluted with water, concentrated, and examined by two-dimensional paper chroma-

**TABLE 2**

<table>
<thead>
<tr>
<th>Nucleoside sugar</th>
<th>Additions ((\mu)moles)</th>
<th>Incorporation ((\mu)moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-Xyl-(^{14})C (boiled control)</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
<td>UDP-Xyl-(^{14})C</td>
<td>-</td>
<td>90.0</td>
</tr>
<tr>
<td>UDP-Xyl-(^{14})C</td>
<td>MgCl(_2) (20)</td>
<td>97.0</td>
</tr>
<tr>
<td>UDP-Gal-(^{14})C (boiled control)</td>
<td>-</td>
<td>0.85</td>
</tr>
<tr>
<td>UDP-Gal-(^{14})C</td>
<td>MgCl(_2) (20)</td>
<td>207.0</td>
</tr>
<tr>
<td>UDP-Gal-(^{14})C</td>
<td>UDP-Xyl (0.5)</td>
<td>245.0</td>
</tr>
<tr>
<td>UDP-Gal-(^{14})C</td>
<td>UDP-Xyl (0.5)</td>
<td>296.0</td>
</tr>
<tr>
<td>UDP-Gal-(^{14})C</td>
<td>MgCl(_2) (20) + UDP-Xyl (0.5)</td>
<td>312.0</td>
</tr>
</tbody>
</table>

Epiphyses from 45 embryo chicks were homogenized in 20 ml of buffer B containing 0.01 M MnCl\(_2\) and 0.05 M Tris-acetate buffer pH 6.0. Each tube contained 2.0 ml of the 10,000-g supernatant fraction, additions as shown in the table, and either UDP-Xyl-\(^{14}\)C (\(9.3 \mu\)moles, 0.21 \(\mu\)c) or UDP-Gal-\(^{14}\)C (15.7 \(\mu\)moles, 0.27 \(\mu\)c) in a total volume of 2.05 ml. After 60 min at 37°C the reactions were stopped by immersion of the tubes in a boiling water bath for 3 min, and the protein was precipitated with TCA and assayed for radioactivity as outlined in Methods.
tography in solvents G and C, respectively. Radioactivity (ca. 1000 cpm) was localized by autoradiography using Kodak Blue-brand X-ray film (exposure time, three weeks). A single radioactive spot was found which corresponded exactly in shape and position to the ninhydrin color of a xylosylserine internal standard.

![Image](image_url)

**Fig. 4.**—Paper chromatogram of xylitol-C\(^{14}\) (solid line) and galactose-C\(^{14}\)-labeled material (broken line) obtained by treatment of xylose-C\(^{14}\) and galactose-C\(^{14}\)-labeled proteins separately with alkaline borohydride. The substances were applied to Whatman 3 MM paper and subjected to descending paper chromatography in solvent D for 48 hr. The blackened areas correspond to sugar standards stained with alkaline silver nitrate. Gal-xyl represents a galactosyl-xylose standard. The radioactive paper strips were cut into 1-cm sections and assayed for radioactivity as described in Methods.

**Formation of DNP-xylosylserine:** A separate sample (10,000 cpm) of the crude xylosyl-C\(^{14}\)-serine, eluted from Dowex 50 with 2 M pyridine, was mixed with 0.5 µmole of authentic xylosylserine, and the DNP derivative was prepared as described in Methods. DNP-xylosyl-C\(^{14}\)-serine was characterized by paper chromatography in solvents B and E. The DNP derivative was not pure, but in each solvent, 30–40 per cent of the radioactivity was found in a sharp peak coinciding in position with the yellow color of authentic DNP-xylosylserine.

**Incorporation of galactose-C\(^{14}\):** The 10,000 g supernatant preparation from embryonic chick cartilage also catalyzed the transfer of galactose-C\(^{14}\) from UDP-Gal-C\(^{14}\) to TCA-precipitable protein. Figure 1 shows that the optimum pH for this reaction was 5.5. The effect of manganous and magnesium ion concentration is shown in Figure 2. Manganese causes a fivefold increase in incorporation, whereas magnesium had little effect.

Since galactose occurs linked to xylose in the CS-protein complex, the effect of added UDP-xylose on the incorporation of galactose-C\(^{14}\) was investigated. The results in Table 2 indicate that the extent of galactose incorporation was not altered by the addition of UDP-Xyl under any of the conditions employed.

**Characterization of the galactose-C\(^{14}\)-labeled product:** Because galactose is a constituent of many glycoproteins and occurs covalently bound to collagen,\(^{23}\) \(^{24}\) it was essential that the galactose-labeled product be well characterized. In order to
demonstrate that the incorporation was primarily into noncollagenous protein, a labeled TCA precipitate (11 mg, 15,000 cpm) was extracted with 0.5 M Tris-acetate buffer (pH 8.0) at 37$^\circ$ for 48 hours. The insoluble material was removed by centrifugation, and the supernatant solution (6,450 cpm, 43%) was subjected to gel filtration on Sephadex G-50 (superfine beads, 1.2 $\times$ 113 cm), in 0.05 M Tris-acetate buffer, pH 8.0. The radioactivity emerged in a single peak which was not retarded by the gel. This material was divided into two aliquots; one was digested with pronase and the other with purified collagenase, following which each digest was subjected to gel filtration as before. The pronase digest yielded a small radioactive peak in the void volume, but most of the radioactivity was eluted in two broad retarded peaks. In contrast, the collagenase digest yielded only one radioactive peak in the void volume. These data establish that the galactose is attached to a non-collagenous protein.

**Alkali lability of the galactose-labeled protein:** Figure 3 shows that mild alkali treatment of galactose-C$^{14}$-labeled protein solubilizes approximately 75 per cent of the radioactivity, as might be expected if the galactose were linked, in part, to protein through an alkali-labile xylose residue. To confirm this, a separate sample of the galactose-C$^{14}$-labeled protein (56,700 cpm) was treated with alkali and borohydride as outlined in Methods. The solution containing the radioactivity solubilized by the alkali (38,200 cpm, 67%) was desalted with Dowex resins (Methods), and the resulting material (25,000 cpm, 44%) analyzed by paper chromatography in solvent D. All of the radioactivity moved as a single peak slightly ahead of galactosylxylose, but well behind the galactose or galactitol standards (Fig. 4). A sample of the material was subjected to acid hydrolysis in 2.0 N HCl at 100$^\circ$ for 30 minutes and analyzed by paper chromatography in the same solvent. The radioactivity moved as does free galactose and not as galactitol. This result might be expected if the compound liberated by the alkaline borohydride were galactosyl-C$^{14}$-xylitol. Another sample of the galactose-C$^{14}$-protein was treated with 2.5 ml of 0.5 N KOH which contained 20 mg of KBH$_4$ and 10 mg of NaBH$_4$-H$^3$ (137 mc/m mole). The galactosylxylitol was isolated as described above and purified by preparative paper chromatography in solvent D. After hydrolysis of this material by 2 N HCl, galactose-C$^{14}$ was again demonstrated by chromatography with solvent D. The tritium was distributed in a number of spots, which have not as yet been identified. Treatment with $\beta$-galactosidase resulted in liberation of a significant portion of free galactose-C$^{14}$. These results indicate that the transferred galactose-C$^{14}$ is present in an alkali-stable bond, a significant portion of which has a $\beta$-configuration.

**Discussion.**—Recent studies from this laboratory have indicated that protein synthesis is a prerequisite for the formation of polysaccharide chains in the biosynthesis of CS-protein complex.25 Polysaccharide chains are formed by stepwise addition of glucuronic acid and N-acetylgalactosamine residues to the nonreducing end of the molecule.10 In view of the structure of the protein-polysaccharide linkage region, it might be anticipated that after synthesis of the protein moiety of the complex, and prior to polymerization of the CS chain, one xylose and two galactose residues are added sequentially to appropriate serine residues.

The data presented in this paper indicate that cell-free preparations from embryonic chick cartilage do indeed catalyze the transfer of xylose to serine residues of TCA-insoluble material. It is clear that the reaction is not due to the transfer of
xylose to free serine which subsequently is incorporated into protein since addition of the usual substrates necessary for cell-free protein synthesis did not stimulate the incorporation of xylose. Furthermore, no xylosylserine-C\textsuperscript{14} could be detected when UDP-Xyl, ATP, and serine-C\textsuperscript{14} were incubated with a 10,000 g supernatant fraction.

The cell-free system also catalyzes the incorporation of galactose into TCA-insoluble material. Treatment of the labeled product with alkaline borohydride results in release of radioactivity. Free galactitol-C\textsuperscript{14} could not be demonstrated, but rather an unidentified compound which in turn yields free galactose-C\textsuperscript{14} after hydrolysis by acid or β-galactosidase. Although linkage between the incorporated galactose and xylose has not been demonstrated, this evidence indicates that a portion of the galactose is linked to another residue—possibly xylose—by a β-galactosidic bond that is stable to alkali.

It would be reasonable to assume that the crude enzyme preparation contains the appropriate acceptor protein, to which xylose, galactose, and finally the CS chains are added. Other studies, however, suggest that the mechanism may be more complicated. Treatment of minced cartilage with puromycin\textsuperscript{25} before preparation of the cell-free enzyme might be expected to decrease the concentration of such an acceptor protein.\textsuperscript{26} Unexpectedly, enzyme prepared from puromycin-treated tissue was fully active in the incorporation of xylose. This result raises the possibility that the xylose incorporation observed in these experiments reflects the formation of an intermediate. Such intermediates have been demonstrated in the biosynthesis of complex polysaccharides in bacteria.\textsuperscript{27}–\textsuperscript{29}

The nature of the xylose acceptor and the mechanism of addition of the two galactose units and the first uronic acid residue remain to be elucidated.

**Summary.**—A cell-free preparation from embryonic chick cartilage catalyzes the incorporation of xylose-C\textsuperscript{14} and galactose-C\textsuperscript{14} from the corresponding UDP derivatives into TCA-precipitable material. The xylose is incorporated without modification and can be entirely solubilized by treatment with alkaline borohydride yielding xylitol-C\textsuperscript{14}. Xylosyl-C\textsuperscript{14}-serine was isolated after extensive proteolytic digestion. Free galactose-C\textsuperscript{14} can be recovered following acid and β-galactosidase hydrolysis of radioactive products isolated by alkali treatment of galactose-C\textsuperscript{14}-labeled TCA precipitates.

It is proposed that these results reflect the biosynthesis of the linkage region of CS to protein.

*Note added in proof:* The product obtained by the treatment of galactose-C\textsuperscript{14}-labeled protein with alkaline borohydride appears to be 4-O-β-D-galactosyl-D-xylitol. It had a chromatographic mobility identical with a known sample of the latter in three solvents.

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\textsuperscript{5} Lindahl, B., E. Stern, and L. Rodén, personal communication.
7 Mathews, M. B., personal communication.