PHOTOAFFINITY ANALOG OF HERBICIDE INHIBITING CELLULOSE BIOSYNTHESIS: Synthesis of [^{3}H]-2,6-Dichlorophenylazide

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Summary:
The compound 2,6-dichlorobenzonitrile (dichlobenil, DCB) is known to be a reversible inhibitor of in vivo cellulose biosynthesis in higher plants and algae. We now report the synthesis of 2,6-dichlorophenylazide (DCPA) with a non-exchangeable tritium label of high specific activity. We have shown this compound to compete with DCB in binding to cotton fiber extracts, and to specifically label a low molecular weight protein in this extract on irradiation with UV light.

Introduction:

Although cellulose is one of the world's most abundant natural products, the enzymes catalyzing its biosynthesis are poorly characterized. In vitro cellulose biosynthesis was carried out effectively for the first time very recently (1).

The compound 2,6-dichlorobenzonitrile (DCB, dichlobenil), long used as a commercial herbicide, has been shown to inhibit cellulose biosynthesis (2). The initial target of DCB is unknown, but it must play a key role in the polymerization of glucose in vivo. Identification of the DCB receptor and characterization of its normal function in biosynthesis would be useful in more fully understanding this system.

For this reason, we undertook to label and isolate the DCB receptor. Examining the structure of DCB, it was clear that the benzonitrile moiety was similar in terms of steric and electronic factors to an aryl azide. As aryl azides are also well known to be useful as photoaffinity labels, we proposed that 2,6-dichlorophenylazide (DCPA) should be an inhibitor of DCB binding, competitive in the dark and irreversible after UV irradiation. It also appeared possible that DCPA might itself block cellulose biosynthesis. Synthesizing this compound with a non-exchangeable radiolabel should then allow us to detect and purify the covalently modified receptor after photoaffinity labelling.

The biological results, which are described elsewhere (3), confirm that DCPA is a useful photoaffinity analog of DCB, specifically labelling a polypeptide with a molecular weight of about 18 kilodaltons in cotton fiber extracts pre-incubated with DCPA then exposed to short-wave UV irradiation. DCPA also inhibits cellulose biosynthesis (3) and is toxic to whole plants (4). Although DCPA is a known compound (5), we are not aware of any reported biological activity. In the present work, we report the synthesis and purification of tritiated DCPA.

Experimental Procedures:

Synthesis of unlabelled DCPA was carried out in a one-pot procedure by diazotization of 2,6-dichloroaniline in a two-phase solvent mixture, followed by reaction of the unisolated diazonium salt with azide ion.

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Several approaches were evaluated for introduction of a non-exchangeable tritium label. Initially, we examined the reductive dehalogenation of 4-iodo-2,6-dichloroaniline. This compound was prepared by treatment of 2,6-dichloroaniline with iodine monochloride in acetic acid. Using conditions analogous to those that would be used with tritium gas, we attempted to cleave the iodo group with both platinum and palladium catalysts under one atmosphere pressure of hydrogen. Even several days exposure resulted in less than 10% conversion to 2,6-dichloroaniline.

We then arranged to have New England Nuclear Corp. carry out non-specific introduction of tritium using carrier-free tritium oxide and a catalyst. Treatment of dichloroaniline with tritium oxide in triethylamine over 5% rhodium/alumina yielded a product which was found to contain less than 1% of 2,6-dichloroaniline as determined by co-chromatography with a cold sample of the compound. However, use of trifluoroacetic acid in place of triethylamine resulted in a cleaner reaction . After removal of exchangeable tritium with ethanol, and of trifluoroacetic acid with sodium carbonate, HPLC of this material showed that about 20% of the total radioactivity co-chromatographed with cold carrier 2,6-dichloroaniline. The crude specific activity was 52.3 mCi/mmole (assuming a scintillation counting efficiency of 33%). A sample of this material was purified by preparative HPLC to yield 13.0 mg (80 μmoles) of 2,6-dichloroaniline.

A portion of this was converted to the corresponding azide as described below. The crude azide was purified by HPLC to yield the material used for biological testing. The specific activity of the purified azide was about 2.4 Ci/mmole.

Synthesis of cold 2,6-dichlorophenylazide (DCPA):

A sample of 2,6-dichloroaniline (Aldrich, 270 mg, 1.67 mmole) was dissolved in hexane (6 ml), and a mixture of water (5 ml) and 5N sulfuric acid (1.65 ml) was added. Sodium nitrite (260 mg, 3.06 mmole) in water (4 ml) was added dropwise with the flask cooled in an ice bath. After an hour at ice temperature, sodium azide (130 mg, 2.0 mmole) was added. After an additional hour, the organic phase was separated, washed with 5% aqueous sodium carbonate, dried (MgSO4) and the solvent stripped to yield 180 mg (0.96 mmole, 57%) 2,6-dichlorophenylazide in pure form.

NMR (H, CDCl3): δ7.25 (d) 2H, δ7.00 (t) 1H, J = 8 Hz.
UV (ethanol): λ_{max} = 247 nm, ε = 5725.
IR (CHCl3, cm-1): 2150, 2100, 1440, 1300.

Synthesis and purification of tritiated DCPA:

A sample of 2,6-dichloroaniline (30.2 mg, 0.18 mmole) was dissolved in trifluoroacetic acid (0.3 ml) plus carrier-free tritium oxide (9μl, 25 Ci), and 5% rhodium/alumina (100 mg) was added. The mixture was stirred overnight at 80 degrees C. Excess water was removed under vacuum, ethanol added, the catalyst filtered off, and the filtrate was evaporated to dryness then redissolved in ethanol (10 ml). A total of 49.5 mCi was contained in this solution. The ethanol solution was treated with anhydrous sodium carbonate (100 mg) for 5 minutes to remove trifluoroacetic acid, and filtered through anhydrous magnesium sulfate, which was washed with ethanol. The volume of the ethanol solution was reduced to 10 ml under nitrogen stream.

A 10 μl portion of this solution was mixed with cold dichloroaniline (35 mg) in ethanol (1.5 ml), and was subjected to HPLC on a Varian Micro-Pak MCH-10 30x0.4 cm column using solvent gradient elution from 75% methanol/25% water to pure methanol over a 10 minute period. Fractions were collected and counted. The carrier dichloroaniline, with a retention time of about 7.5 minutes, co-eluted with 26.5% of recovered counts, or 21.3% of total counts placed on the LC column.

Approximately one half of the original radioactive sample was purified by preparative HPLC using the same column and conditions as above. A fraction eluting at 7.5 minutes (corresponding to the center of the dichloroaniline UV absorptivity peak) was collected from the repetitive injections, and the pooled material was diluted with methanol to a final volume of 100 ml.

The bulk of the methanol solution was reduced under nitrogen stream to about 2 ml. Sulfuric acid (1.0 ml of 2.5 M) was added, the solution cooled in ice, methylene chloride (2.0 ml)was added, followed by sodium nitrite solution (1.0 ml of 2.0 M). The solution was stirred at ice temperature 15 minutes in the dark. Sodium azide

solution (1.0 ml of 2.0 M) was added, the solution stirred one hour in the ice bath, then the organic phase was separated, the aqueous phase washed with two portions of methylene chloride, the combined organic phase dried (MgSO4), and the volume reduced to 1.0 ml under nitrogen stream.

A portion of the azide solution was purified by preparative HPLC using the same conditions as above. The fraction that eluted between 12.4 and 14.7 minutes retention time was collected. This fraction was found to contain 7.7 µgm (41 nmoles, 74 Mcpm, 99.0 µCi), and accounted for 94% of the total counts placed on the HPLC column. A sample of this fraction was found to co-elute with cold carrier DCPA. This material, which had a specific activity of 2.4 Ci/mmole, was used directly in the biological studies.

NMR spectra were obtained in $CDCl_3$ on a Varian XL-300, and are reported in delta values. IR spectra were taken on a Nicolet 10-MX FT-IR. UV spectra were obtained using a Cary 219. Tritium counting was carried out using a Beckmann LS7500 in Scintosol (Isolab). HPLC analyses and purifications were performed on a Hewlett-Packard 1084B machine.

Results and Discussion:

As described in detail elsewhere (3), DCPA was tested as an inhibitor of cellulose biosynthesis in vivo, an inhibitor of DCB binding in the dark in vitro, and as a photoaffinity label. By measuring incorporation of [^{14}C]-glucose into cellulose in cultured cotton ovules, it was shown that the effect of DCPA without exposure to UV light closely paralleled that of DCB, causing a 50% inhibition of cellulose biosynthesis at a concentraion of 1.0 µM. At a concentration of 10 µM, both DCB and DCPA reduced total incorporation of glucose into cellulose by more than 80%.

When DCPA was incubated with extracts of cotton fibers, then exposed to short-wave UV irradiation, incorporation of tritium label into a protein fraction was observed. After this treatment, polyacrylamide gel electrophoresis of SDS-denatured total cotton fiber proteins showed that selective labelling of a protein with a molecular weight of about 18 kilodaltons occurred. This selective labelling was blocked by the presence of DCB. More extensive structural studies of the labelled peptide are underway.

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