SYNTHESIS OF ^{14}C-INDOLE-3-ACETYL-*MYO*-INOSITOL

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SUMMARY

Synthesis of the mixed isomeric ^{14}C-indoleacetyl-*myo*-inositols from carrier-free β[2-^{14}C]-indoleacetic acid (57.2 mCi/mmole) and inositol *via* an imidazolide intermediate is described. Radiological decomposition of the indolylic compounds was prevented by the use of a volatile thiol, dithioethane, and anthracene.

Key Words: Dithioethane, Indole-3-acetic Acid, Indole-3-acetyl-*myo*-inositol, Inositol

INTRODUCTION

Esters of the plant growth hormone indole-3-acetic acid (IAA) and inositol or inositol glycosides comprise the bulk of the water soluble IAA found in seeds of corn (*Zea mays*) (1). These complexes are synthesized by a CoASH and ATP dependent enzyme system (2) and are hydrolyzed to free IAA by autolyzing plant tissue (3). The hormonal conjugates play a role in hormonal homeostasis (4,5), protection against peroxidase oxidation (S and J. Cohen, unpublished), and transport of hormone within the plant (S and J. Nowacki, unpublished). Further studies of these metabolic functions would be facilitated by the availability of isotopically labeled esters.

Labeled indoleacetyl-*myo*-inositol would also serve as a standard for quantitative studies of IAA-esters by isotope dilution (6). Free IAA is not the ideal internal standard for determination of total plant IAA since the cereals, for example, contain 90% ester IAA (7). Free IAA and esterified IAA differ in stability in plant extracts since IAA is oxidized by peroxidase while the ester forms are resistant (S and J. Cohen, unpublished). The ester and free forms of IAA may also exhibit different chemical stability since, for example, Felker (8) has shown that free tryptophan was more stable than peptidically linked tryptophan during acid hydrolysis of protein. For these

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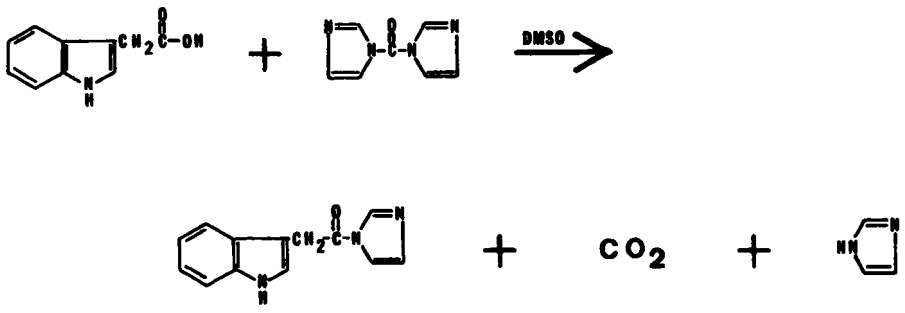
reasons, isotopically labeled indoleacetyl-*myo*-inositols would be a good internal standard for the quantification of either total plant IAA or ester IAA.

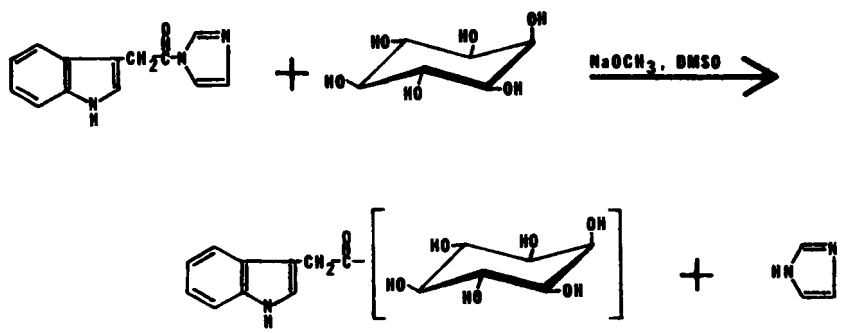
DISCUSSION

The reactions used to synthesize the indoleacetyl-*myo*-inositols are shown in scheme 1 (reactions 1 and 2). This is essentially the generalized method for ester synthesis described by Staab (9) involving the formation of the imidazolide intermediate. It was chosen over more conventional methods because of the mild reaction conditions used. No attempt was made to synthesize a particular axial or equatorial ester since under our conditions of usage acyl migration rapidly produces all four of the chemically resolvable indoleacetyl-*myo*-inositols (10).

1.



2.



β[2-^{14}C]-Indoleacetic acid, specific activity 57.2 mCi/mmole, was obtained from New England Nuclear and used without isotopic dilution. The ^{14}C-IAA was purified prior to use by chromatography on a 2 x 22 cm column of Sephadex LH-20 with elution by ethanol-water (1:1). The volatile thiol reagent, dithioethane (Aldrich Chemical Co., Milwaukee, Wisc.), was added (10 μmole/μmole ^{14}C-IAA) to protect the ^{14}C-IAA from oxidation (cf. 8). The ^{14}C-IAA fraction was evaporated to dryness under nitrogen in the presence of 0.56 μmole of anthracene (J. T. Baker,

Phillipsburg, New Jersey) per μmole of ^{14}C-IAA. Anthracene acted to protect the radioactive IAA during the drying step and its presence had no effect on subsequent reactions. Attempts at purification without use of dithioethane and anthracene resulted in almost complete loss of the ^{14}C-IAA. Purification of ^{14}C-IAA prior to use was obligatory.

Stoichiometric, rather than catalytic, amounts of sodium methoxide were found to be necessary, possibly because of the difficulty in abstracting a proton from the cyclitol. Sodium methoxide was prepared under dry nitrogen by adding freshly cut sodium metal to anhydrous methanol (11). Methanol was twice redistilled from magnesium activated with iodine (12) onto heat activated Linde 4 A (Supelco, Bellefonte, Pennsylvania) and used immediately. Residual methanol was removed from sodium methoxide *in vacuo* at 60°C and then by drying for 18 hr at 200°C under high vacuum. This product was stored *in vacuo* over P_2O_5 in a dry nitrogen flushed desiccator. Even small amounts of water resulted in the formation of enough NaOH to hydrolyze the esters as they were made. It was necessary to add sodium methoxide to the reaction as a dry powder since solutions of sodium methoxide in dimethylsulfoxide (DMSO) were ineffective.

myo-Inositol (Sigma Chimical Co., Saint Louis, Missouri) was dried *in vacuo* in an Abderhalden apparatus at 100°C for 18 hr. DMSO was freshly distilled *in vacuo* onto Linde 4A. DMSO was stored in sealed 1 ml vials over Linde 4A and the solvent was obtained through a teflon septum as needed. These vials were stored over anhydrous $CaSO_4$ and discarded once the septum had been perforated. All glassware was dried at 105°C overnight prior to use. Manipulations involving sodium methoxide were carried out in a glove box in a dry nitrogen atmosphere with P_2O_5 present as desiccant.

The identity of the radioactive product was established by a number of criteria. First, a parallel synthesis was run on a larger scale using unlabeled IAA and the identity of the products of this synthesis was confirmed as follows. The synthetic products had the same R_f values on thin layer chromatograms (E. Merck, Darmstadt, silica gel plates developed in methyl ethyl ketone-ethyl acetate-ethanol-water (3:5:1:1)) as did the naturally occurring mixture of axial and equatorial IAA-inostitols (1). They gave positive reactions with Ehmann's reagent (13) as did the indoleacetyl-*myo*-inositols isolated from corn seeds. Ammonolysis in 14% NH_4OH for 30 min at 45°C yielded two products, one of which co-chromatographed with authentic IAA and the other with indoleacetamide. The products had the characteristic elution volume on a high pressure liquid column (14) and the correct retention times when subjected to gas liquid chromatography (2). Combined gas chromatography/mass spectrometry of any of the four resolvable fully trimethylsilyated derivatives (10) using a LKB 9000 mass spectrometer yielded a molecular ion at m/e = 769 and a fragmentation pattern identical to that previously published for the axial and equatorial esters (10,15). The ^{14}C compounds were then compared with the unlabeled compounds. The ^{14}C compounds yielded labeled IAA and indoleacetamide upon ammonolysis. Their R_f values on thin layer chromatograms and their

elution volumes from a high pressure liquid chromatographic column were identical to those of the unlabeled isomeric indoleacetyl-*myo*-inositols.

EXPERIMENTAL

After chromatography on Sephadex LH-20, β[2-^{14}C]-indoleacetic acid (210 μg, 1.2 μmoles, 68.6 μCi) yielded 175 μg (1 μmole) of purified product. The pooled fractions were evaporated to a small volume on a rotary evaporator and 100 μl hexane containing 100 μg of anthracene was added. The mixture was dried under nitrogen in a 300 μl microflex tube (Kontes, Vineland, New Jersey).

1,1'-Carbonyldiimidazole (Sigma; 324 μg, 2 μmoles) in 10 μl dry DMSO was added to the dry residue through the septum of the microflex tube and mixed vigorously. Three 1 mm glass beads (B. Braun, Melsungen) were added to aid in dissolving the dry residue. After 30 min of incubation at 25°C, inositol (540 μg, 3 μmoles) in 60 μl DMSO was added with a syringe. The tube was then placed in a nitrogen-flushed dry box and a small amount of sodium methoxide (about 1 μmole) was added as a dry powder. The reaction mixture was mixed vigorously for 1-2 min and the reaction stopped by adding 100 μl of ice-cold 2-propanol-4 M acetic acid (1:1).

Immediately after termination of the reaction the 170 μl of reaction mixture was applied to a 0.9 x 17 cm high pressure column of sulfonated styrene-divinylbenzene copolymer (Beckman PA-28) and separated using 2-propanol-water (1:1) as the mobile phase (14). Fractions containing significant radioactivity eluted as expected for the isomers of indoleacetyl-*myo*-inositol and these were pooled. Alcohol was removed *in vacuo* and the water phase lyophilized onto 100 mg of cellulose powder. The products were eluted from the cellulose with 1 ml ethanol-water (1:1).

The yield was calculated from a 5 μl sample of the final solution based on the specific radioactivity of the sample. The yield was 76 μg of ^{14}C-indoleacetyl-*myo*-inositols (13 μCi, 23%) for this synthesis.

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REFERENCES

1. Bandurski R. S., Ueda M., and Nicholls P. B. – Ann. N. Y. Acad. Sci. *165*: 655 (1969)
2. Kopcewicz J., Ehmann A., and Bandurski R. S. – Plant Physiol. *54*: 846 (1974)
3. Hamilton R. H., Bandurski R.S., and Grigsby B. H. – Plant Physiol. *36*: 354 (1961)
4. Bandurski R. S. and Schulze A. – Plant Physiol. *59(S)*: 10 (1977)

5. Bandurski R. S. – In "Cyclitols and the Phosphoinositides," Wells, W. W. and Eisenberg, F., editors, Academic Press, New York, 1977, in press

6. Bandurski R. S. and Schulze A. – Plant Physiol. *54*: 257 (1974)

7. Bandurski R. S. and Schulze A. – Plant Physiol. *60*: 211 (1977)

8. Felker P. – Anal. Biochem. *76* 192 (1976)

9. Staab H. A. – Angew. Chem. internat. Edit. *1*: 351 (1962)

10. Ehmann A. and Bandurski R. S. – Carbohyd. Res. *36*: 1 (1974)

11. Burness D. M. – Organic Syntheses *39*: 49 (1959)

12. Riddick J. A. and Bunger W. B. – "Organic Solvents – Physical Properties and Methods of Purfication," Wiley-Interscience, New York, 1970

13. Ehmann A. – J. Chromatogr. *132*: 267 (1977)

14. Cohen J. D. and Bandurski R. S. – Plant Physiol. *59(S)*: 10 (1977)

15. Ehmann A., Bandurski R. S., Harten J., and Sweeley C. C. – "Mass Spectrometry of Indoles and Trimethylsilyl-indole Derivitives," Michigan State University, East Lansing, Michigan, 1975