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An Acetyl-Co A: Cytisine N-Acetyltransferase in *Sophora* Seedlings[1]

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N-Acetylcytisine (**2**) was formed by an enzyme in *Sophora* seedlings from cytisine (**5**) in the presence of acetyl-coenzyme A. An acetyl-coenzyme A: cytisine N-acetyltransferase was reasonably stable upon a heat treatment at 90° for 15 min. Some other properties of the enzyme are described.

Keywords——acetyl-Co A: cytisine N-acetyltransferase; lupin alkaloid; cytisine; N-acetyl-cytisine; acetyl-coenzyme A; enzyme; N-acetyltransferase; *leguminosae*; *Sophora tomentosa*; *Sophora flavescens*

We have previously isolated N-formylcytisine (**1**),[3] N-Acetylcytisine (**2**)[4] and N-(3-oxo-butyl)cytisine (**3**)[5] from the genus *Thermopsis*, *Sophora* and *Echinosophora*, respectively, in *leguminosae*. **1** and **3** are new lupin alkaloids so far found in nature.

Although N-substituted cytisine derivatives such as **1**, **2**, **3** and N-methylcytisine (**4**) are widely distributed in *leguminosae* as minor components generally, research on the origin of the N-substituted side chain of cytisine derivatives is limited. However, our recent work with enzyme system prepared from *Thermopsis* and *Lupinus* seedlings has indicated that the N-methyl group of N-methylcytisine is derived from L-methionine as a donor in the form of S-adenosyl-L-methionine.[6]

The present work reports an evidence for the participation of acetyl-coenzyme A (acetyl-Co A) in the formation of N-acetylcytisine (**2**) in *Sophora* seedlings.

Materials and Methods

Plant Materials——The seedlings of *Sophora tomentosa*, *Sophora flavescens*, *Thermopsis chinensis* and *Lupinus luteus* were grown in moistened vermiculite in the dark for 4—6 days at 30°. After harvest, the testas were removed and the seedlings were cooled at 0° for 30 min before extraction.

Chemicals——Acetyl-coenzyme A was purchased from Sigma Co. Cytisine (mp 154—155°, $[\alpha]_D^{19}$ $-121°$) was isolated from *Sophora* and *Thermopsis* species as described in the previous papers.[3,4]

Enzyme Preparation——Plant materials were macerated at 0—4° in a cold mortar with a small amount of Si sand in the presence of 0.05 M K-phosphate buffer, pH 7.8, containing 0.1% (v/v) 2-mercaptoethanol (0.5 ml/g of plant materials). To the homogenates was added polyclar AT (0.1 g/g of tissue) and after stirring for 20 min the mixtures were squeezed through fine nylon and centrifuged for 30 min at 25000 ***g***. The protein precipitate obtained from the supernatants by the addition of $(NH_4)_2SO_4$ at 10 to 75% saturation

1) This work was presented at the 97th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1977.
2) Location: *a) 1–33 Yayoi-cho, Chiba*; *b) 2–4–41, Ebara, Shinagawa-ku, Tokyo.*
3) S. Ohmiya, H. Otomasu, I. Murakoshi, and J. Haginiwa, *Phytochemistry*, 13, 643 (1974).
4) S. Ohmiya, H. Otomasu, I. Murakoshi, and J. Haginiwa, *Phytochemistry*, 13, 1016 (1974).
5) I. Murakoshi, K. Fukuchi, J. Haginiwa, S. Ohmiya, and H. Otomasu, presented at the Meeting of Kanto Branch, Pharmaceutical Society of Japan, Tokyo, November, 1976: *Phytochemistry*, 16, 1460 (1977).
6) I. Murakoshi, A. Sanda, J. Haginiwa, N. Suzuki, S. Ohmiya, and H. Otomasu, presented at The Meeting of Kanto Branch, Pharmaceutical Society of Japan, Tokyo, November, 1976: *Chem. Pharm. Bull.* (Tokyo), 25, 1970 (1977).

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was dissolved in the minimum volume of the extracting buffer and the clear supernatant solution, recovered by centrifugation at 25000 *g* for 10 min, was applied to a column of Sephadex G-25 (fine), previously equilibrated with the extracting buffer, to give a protein-containing solution free from low mol. wt. substances, which was used directly as the crude enzyme (stage 1) in preliminary experiments.

Further purification yielding a highly active protein was achieved by a heat treatment: the protein solution of stage 1 was brought to 90° in boiling water bath with vigorous stirring and held at 90—92° for an additional 15 min. After cooling immediately to 4° with ice-water, the precipitated protein was removed by centrifugation at 15000 *g* for 20 min. The resulting supernatant (stage 2) was subjected to the assay for N-acetyltransferase activity in most experiments. Unless otherwise specified, enzyme preparations (stage 2) obtained from the seedlings of *Sophora tomentosa* in which N-acetylcytisine (2) occurred were normally used as the source of enzyme activity.

Assay of Acetyl-Co A: Cytisine N-Acetyltransferase Activity——The normal reaction mixtures contained cytisine (5 μmol), acetyl-Co A (1 μmol) and 0.4 ml of enzyme preparation (stage 2), containing 1.62 mg of the soluble protein from about 10 g fresh weight of seedlings, in a final volume of 0.6 ml. Reaction mixtures were normally maintained at pH 8.0 by 0.05 M Tris-HCl buffer. Reactions were conducted at 30° for 30 min and terminated by the addition of a few drops of conc. HCl. With each batch of samples, the control tubes, from which acetyl-Co A, cytisine or enzyme preparation had been omitted, were included.

Identification of N-Acetylcytisine as the Reaction Product——The acid reaction mixtures terminated with HCl were extracted twice with CH_2Cl_2 and then made alkaline with conc. K_2CO_3 solution. The alkaloids were extracted from the resulting alkaline solution with CH_2Cl_2. The formation of N-acetylcytisine was confirmed by subjecting the alkaloidal fraction to TLC on silica gel G (Merck, type 60) and high-speed liquid chromatography (Kyowa Seimitsu, Model K-880) employing a monitoring flow system (310 nm). The reaction product co-chromatographed with authentic N-acetylcytisine, isolated from *Sophora tomentosa*,[4] in the following solvent systems: 1, $CHCl_3$–MeOH (5: 1, v/v); 2, CH_2Cl_2–MeOH–28%NH_4OH (90: 9: 1, v/v); 3, CH_3CN–MeOH–28%NH_4OH (15: 19.5: 0.5, v/v); 4, acetone–MeOH (1: 2, v/v). The *Rf* values for N-acetylcytisine on TLC in solvent 1, 2, 3 and 4 were 0.51, 0.55, 0.51 and 0.40, respectively, whilst cytisine had the following *Rf* data; 0.48, 0.44, 0.34 and 0.14, respectively. This method indicated clearly the presence of a product, reacting positively with Dragendorff's reagent after spraying with 5% HCl, iodoplatinic acid and iodine vapor as chromogenic reagents, that was inseparable from added authentic N-acetylcytisine. The further identity of the reaction product as N-acetylcytisine and the quantitative estimation of the amount of N-acetylcytisine formed were established by the use of high-speed liquid chromatography employing a monitoring flow system coupled to recorder, using LiChrosorb SI 100 (mean particle size 10 μm, Merck) as an absorbent and 15% MeOH–Et_2O: H_2O: 25%NH_4OH (500: 10: 1, v/v) as a developing solvent.

The enzyme catalyzed formation of N-acetylcytisine was also determined by MS after eluting the product from TLC plates or a column of liquid chromatography as described in the previous paper.[4]

Protein was determined by the method of Lowry, *et al.*[7]

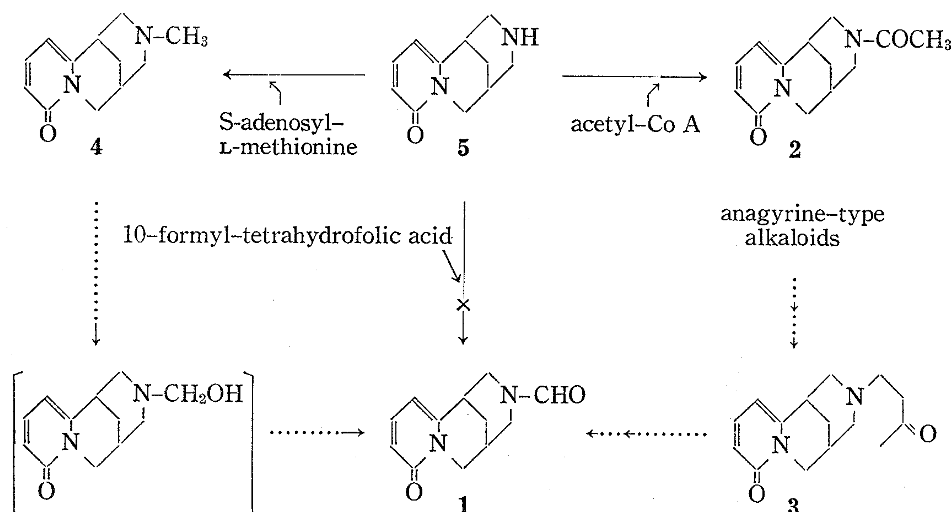

Fig. 1. Scheme for the Biosyntheses of N-Substituted Cytisine Derivatives by Enzymes in the Seedlings of *Sophora* and *Thermopsis* Species

-------: possible biosynthetic pathway.

7) O.H. Lowry, N.J. Rosebrough, A. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

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Results and Discussion

The results presented above demonstrate that acetyl-Co A can be utilized for the acetylation of cytisine (**5**) with formation of N-acetylcytisine (**2**) as shown in Figure 1. Cell free systems were prepared from a number of varieties of *Sophora*, *Thermopsis* and *Lupinus* seedlings in which N-substituted cytisine derivatives occurred, *e. g.*, as shown in Figure 2. In consequence the most active enzyme preparations for cytisine N-acetyltransferase were obtained from *Sophora tomentosa*, but the activity in enzyme preparations from *Thermopsis* and *Lupinus* seedlings was negligible as shown in Figure 3.

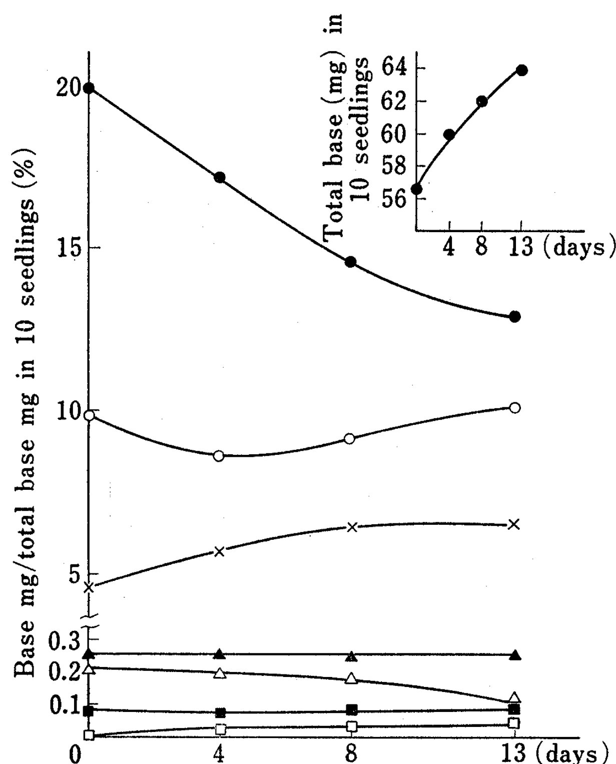

Fig. 2. Variations in Alkaloid Content at Various Stages of Seedling Growth of *Sophora tomentosa* at 30° in the Dark

Alkaloid content was quantitatively estimated by the high-speed liquid chromatography as described in the experimental section. Key: —●—, cytisine; —○—, matrine; —×—, N-methylcytisine; —▲—, baptifoline; —△—, N-formylcytisine; —■—, anagyrine; —□—, N-acetylcytisine.

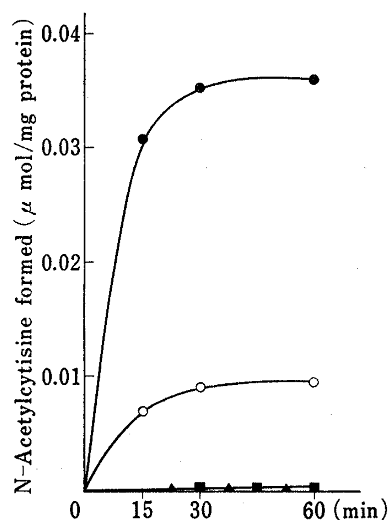

Fig. 3. Comparative Specific Activity for the N-Acetylcytisine Formation by an Enzyme (stage 2) in Seedlings of *Sophora tomentosa* (—●—), *Sophora flavescens* (—○—), *Lupinus luteus* (—▲—) and *Thermopsis chinensis* (—■—)

The seedlings were grown in the dark for 4—6 days at 30°.

The effect of pH on the rate of N-acetylation of cytisine by the enzyme was investigated using 0.05 M Tris-HCl buffer. It was found to have maximal activity at pH 8.0 as shown in Figure 4.

The rate of N-acetylcytisine formation was constant for at least 15 min but the rate then decreased until at 30 min when no further synthesis was detected as shown in Figure 5.

The cytisine N-acetyltransferase was reasonably stable particularly to a heat treatment at 90° for 15—20 min. A remarkable property of the cytisine N-acetyltransferase for a heat treatment was applied to the purification of the enzyme: the specific activity of the partially purified enzyme (stage 2) by ammonium sulphate precipitation between 10 and 75% saturation followed by a heat treatment was approximately 12 fold greater than that of a freshly prepared crude extract (stage 1) as shown in Figure 5.

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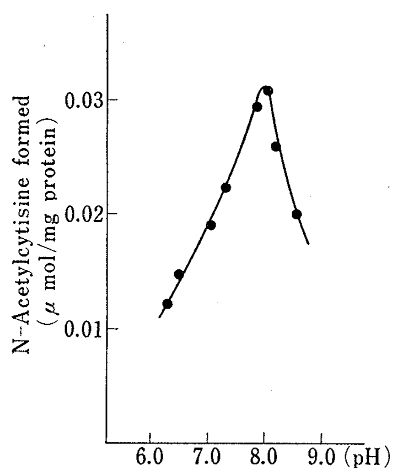

Fig. 4. Effect of pH upon the Cytisine N-Acetyltransferase Activity

Tris-HCl buffer was used at a final concentration of 0.05 M.

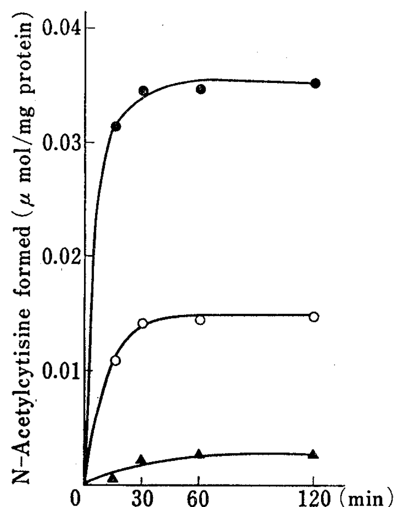

Fig. 5. Comparative Specific Activities of the partially Purified Cytisine N-Acetyltransferase by a Heat Treatment

Key: —▲—, stage 1-enzyme fraction (non-heated); —○—, stage 1-enzyme fraction heated at 70° for 3 min; —●—, stage 1-enzyme fraction heated at 90° for 15 min (stage 2-enzyme fraction).

The specific activity of cytisine N-acetyltransferase decreased by approximately 7 and 79% when the enzyme preparations (stage 2) were pre-incubated with N-ethylmaleimide(1.14 mM) and hydroxylamine (1.03 mM), respectively, as SH-binding reagents, at 30° for 5 min.

The biosynthetic pathways leading to the formations of N-formylcytisine (**1**) and N-(3-oxobutyl)cytisine (**3**) are being undertaken in our laboratories.

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