Note

Biosynthesis of Grayanotoxins in *Leucothoe grayana* Max. Incorporation of Mevalonic Acid and (–)-Kaurene into Grayanotoxin-III

Tetsuya MASUTANI, Masayuki HAMADA,*
Eiko KAWANO,* Junkichi IWASA,**
Zenzaburo KUMAZAWA*** and Hiroo UEDA

Department of Agricultural Chemistry,
University of Osaka Prefecture,
Sakai 591, Japan

*Radiation Center of Osaka Prefecture,
Sakai 593, Japan

**Department of Agricultural Chemistry,
Okayama University,
Tsushima, Okayama 700, Japan

***Department of Agricultural Chemistry,
Mie University,
Tsu, Mie 514, Japan

Received November 13, 1980

Grayanotoxin-I (GTX-I) (1)¹⁾ is the main toxic substance in the young leaves of *Leucothoe grayana* Max. There are a few assumptions on the biosynthesis of grayanotoxins. Iriye *et al.*²⁾ postulated that grayanotoxins may be synthesized from (–)-kaurene on the basis of model chemical reactions. Matsumoto *et al.*³⁾ suggested that grayanols⁴⁾ are the possible biogenetic precursors for grayathol-A⁵⁾ and leucothols,⁶⁾ and even for grayanotoxins. But there has been no experimental evidence concerning the biosynthetic pathways of grayanotoxins. As the first step towards biosynthetic studies on grayanotoxins, we now wish to report the radiochemical evi-

dence for the incorporation of mevalonic acid and (–)-kaurene into grayanotoxins. Sodium DL-[2-¹⁴C]mevalonate was fed to the growing stems of *Leucothoe grayana* Max. by the cotton-wick method. After 3, 6, 9 and 12 days, the stems and leaves were harvested and extracted with methanol. The extract was chromatographed on alumina and silica gel, and GTX-I was separated. For the convenience of chemical and radiochemical purification, isolated GTX-I was diluted with non-radioactive GTX-I and converted to GTX-III (2) by alkaline hydrolysis. GTX-III obtained was recrystallized to constant specific activity. The incorporation of [2-¹⁴C]mevalonate into GTX-III was shown in Table I. The incorporation of [2-¹⁴C]mevalonic acid into GTX-III reaches maximum around 6 days after feeding and the radioactivity in GTX-III seems to decrease gradually thereafter.

(–)-[17-¹⁴C]-ent-Kaurene (3)⁷⁾ prepared was dissolved in acetone-petroleum ether, and applied to the reverse sides of leaves of the growing plant. The leaves were harvested after 12 days. GTX-III was isolated as described above and purified until it showed constant specific activity. From two independent experiments, the incorporation of ¹⁴C-kaurene into GTX-III was 0.0005~0.0007%. Since the radioactivity in the harvested leaves measured after rinsing them with acetone was only about 4.5% of the total activity applied, the low uptake of applied (–)-¹⁴C-kaurene into the leaves might lead to the very low incorporation into GTX-III.

These results imply that grayanotoxins are biologically synthesized *via* mevalonic acid pathway and that (–)-kaurene should be an available precursor for grayanotoxins, although the problem whether or not (–)-kaurene is the direct precursor of grayanotoxins is still unsolved.

EXPERIMENTAL

Radioactivity measurements were performed with Packard Tricarb Liquid Scintillation Spectrometers model 3380 and 460CD. Each sample was dissolved in a scintillation mixture consisting of toluene (10 ml),

TABLE I. RESULTS OF FEEDING EXPERIMENTS OF DL-[2-¹⁴C]MEVALONATE^a

Exp. no	Radio activity fed		Days after feeding	Fresh wt. of raw material harvested (g)	Amount of GTX-I isolated		Activity in GTX-III		Incorpo- ration ratio (%)
	(dpm)	(μmol)			(mg)	(μmol)	(dpm/mg found)	Total activity (dpm)	
1.	8.67 × 10 ⁷	6.7	3	1.8	3.0	7.3	663.0	9282	0.02
2.	8.67 × 10 ⁷	6.7	6	1.8	1.8	4.4	3877.1	49464	0.11
3.	1.30 × 10 ⁸	10.1	9	2.3	2.5	6.1	2685.0	34905	0.05
4.	1.30 × 10 ⁸	10.1	12	2.3	4.0	9.7	3044.3	35923	0.06

^a In all cases, 10 mg of non-radioactive GTX-I was added to GTX-I isolated.

^b These ratios were calculated assuming that only L-mevalonate is physiologically active.

methanol (0.3 ml), PPO (40 mg) and POPOP (1 mg). In each step of recrystallization of GTX-III, radioactivity was counted for 10~100 min.

Feeding of [2-¹⁴C]mevalonate to Leucothoe grayana Max. and isolation of GTX-III. Three drops of 2 N-NaOH was added to DL-[2-¹⁴C]mevalonic acid lactone (0.197 mCi, 5.85 mCi/mmol) and the solution was made up to 5 ml with water. Each 0.5 ml aliquot of the mevalonate solution was fed to each of the 10 stems of *Leucothoe grayana* Max. by the cotton-wick method. After 3, 6, 9 and 12 days, 2, 2, 3 and 3 stems of the plant were harvested, respectively, and macerated with methanol. The methanol extract of the 2 stems (fresh weight: 1.8 g) harvested after 3 days was evaporated to dryness. The residue was dissolved in acetone-methanol (3:1), chromatographed on alumina and eluted with the same solvent system. The eluate thus obtained was further chromatographed on silica gel using benzene containing an increasing concentration of ethyl acetate by 10% steps. Elution with benzen-ethyl acetate (20:80) gave 3.0 mg of GTX-I, which showed a single peak corresponding to GTX-I on TLC. The GTX-I was diluted with non-radioactive GTX-I (10 mg). For the hydrolysis of GTX-I, the combined GTX-I was dissolved in 2%-KOH-50% methanol (1 ml) and warmed at 60°C for 1 hr. The reaction mixture was diluted with saturated NaCl solution, extracted with ethyl acetate. Evaporation of the solvent gave GTX-III as solid, which was recrystallized two or three times from ethyl acetate to constant activity. (total activity, 9282 dpm) The other stems were also treated by use of a similar procedure.

Feeding of (-)-[17-¹⁴C]-ent-kaurene and isolation of

GTX-III. (-)-[17-¹⁴C]-ent-Kaurene (2.567 Ci/mg, 18.4 mg, 67.7 μmol) prepared from (-)-17-nor-ent-kaurene-16-one and ¹⁴C-methyl iodide by Wittig reaction,⁷⁾ was dissolved in 1 ml of acetone-petroleum ether (1:1) and applied to the reverse sides of leaves of the growing plant. The leaves (fresh weight: 6.0 g) were harvested after 12 days. The leaves were rinsed with acetone and macerated with methanol. The leaves after the maceration was assayed by dry combustion method. The sum of the activities of the leaves and methanol extract was about 4.5% of total activity applied. GTX-I was isolated from the extract by the same method as described above. The GTX-I (12.5 mg, 30.3 μmol) was diluted with 10 mg of non-active GTX-I. After hydrolysis of GTX-I, the total activity of GTX-III was 523.2 dpm, and the incorporation of (-)-kaurene into GTX-III was 0.0005%. In another experiment started from 15.15 mg (55.7 μmol) of (-)-[17-¹⁴C]-ent-kaurene, the total activity of isolated GTX-III (5.6 mg, 13.6 μmol) was 595.8 dpm and the incorporation was 0.0007%.

REFERENCES

- 1) S. Miyajima and S. Takei, *Nippon Nôgeikagaku Kaishi*, **10**, 1093 (1934); J. Iwasa, Z. Kumazawa and M. Nakajima, *Chem. & Ind.*, **1961**, 511; J. Iwasa, Z. Kumazawa and M. Nakajima, *Agric. Biol. Chem.*, **25**, 782, 793, 798 (1961).
- 2) R. Iriye and M. Sakakura, *Agric. Biol. Chem.*, **41**, 2109 (1977).
- 3) T. Terai, H. Megri, N. Hamanaka, T. Matsuzaki, A. Furusaki, T. Kato and T. Matsumoto, *Chem. Lett.*, **1980**, 1111.
- 4) S. Fushiya, H. Hikino and T. Takemoto, *Tetrahedron Lett.*, **1974**, 183.
- 5) A. Furusaki, S. Gasa, N. Hamanaka, R. Ikeda and T. Matsumoto, *Chem. Lett.*, **1979**, 665.
- 6) A. Furusaki, N. Hamanaka, H. Miyakoshi, T. Okuno and T. Matsumoto, *Chem. Lett.*, **1972**, 783; H. Hikino, S. Koriyama and T. Takemoto, *Tetrahedron Lett.*, **1972**, 3821.
- 7) B. E. Cross, R. H. B. Galt and J. R. Hanson, *J. Chem. Soc.*, **1964**, 299.