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# Synthesis of high specific radioactivity 3,5-[$^3H_6$]dimethoxy-4-hydroxyacetophenone, an inducing compound of the *vir* Gene in *Agrobacterium tumefaciens*

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## SUMMARY

Acetosyringone (3',5-dimethoxy-4-hydroxyacetophenone, **1**) is one of the plant signals that induce the *vir* genes of *Agrobacterium tumefaciens* which causes crown gall tumors on dicotyledonous plants. *Vir* gene induction is enhanced by other environmental factors like specific monosaccarides and acidic pH. However, it is still unclear how the inducer interacts with *Agrobacterium*. To identify the receptor(s) in *Agrobacterium tumefaciens* to which the plant signal binds, highly radioactive acetosyringone (3,5-[$^3H_6$]dimethoxy-4-hydroxyacetophenone, **6**) was synthesized using $C^3H_3I$ which was generated by two different methods. In Method 1, $C^3H_3I$ was generated from 4-[methyl-$^3$H]methoxy biphenyl to give methyl species with tritium NMR peak intensity ratios ($C^3H_3$ : $C^3H_2H$ : $C^3HH_2$ = 44 : 42 : 14), and a calculated specific radioactivity of 56.1 Ci/mmole. Method 2 gave higher specific radioactivity reagent (72.6 Ci/mmole), with measured NMR peak intensity ratios of ($C^3H_3$ : $C^3H_2H$ : $C^3HH_2$ = 87 : 7 : 6).

**Key Words:** *Agrobacterium tumefaciens*, acetosyringone, tritiomethyl iodide.

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## INTRODUCTION

*Agrobacterium tumefaciens*, a gram negative soil phytopathogen, causes crown gall tumors in a wide variety of dicotyledonous plants. This neoplastic disease is the result of the transfer of a specific DNA segment (T-DNA) localized on the bacterial tumor-inducing (Ti) plasmid to susceptible plant cells (1, 2). The T-DNA transfer is mediated by the expression of a set of virulence (*vir*) genes which are also located on the Ti-plasmid. This *vir* regulon consists of at least ten different operons, *virA* -*virJ* (3, 4). Two of the *vir* genes, *virA* and *virG*, are expressed constitutively at low levels in the absence of plant cells, but the remaining *vir* genes are only expressed in the presence of plant exudates containing phenolic compounds (5, 6). Phenolic plant signals such as acetosyringone or α-hydroxyacetosyringone are excreted from wound sites of the plant and mediate the induction of *vir* regulons through VirA and VirG. The VirA and VirG proteins, products of *virA* and *virG*, have been identified as members of the two-component regulatory systems which respond to environmental stimuli (7). In analogy to other two-component systems, VirA is a membrane-spanning protein which undergoes an ATP-dependent autophosphorylation at a histidine residue in its C-terminus in response to the plant signals (8, 9). The phosphate is transferred to the cytoplasmic protein VirG at an aspartate residue in its N-terminal region. The phosphorylated form of VirG activates the expression of other *vir* genes (10, 11). The *vir* gene induction in response to plant phenolics is enhanced by other environmental signals, such as specific monosaccharides and acidic pH. This sugar-mediated induction requires the ChvE protein, a periplasmic glucose- and galactose-binding protein (12). However, it is still unclear how the plant signal interacts with *Agrobacterium* to initiate the signal transduction process. Although the functions of the VirA and VirG proteins have been characterized well, the interactions between plant phenolic signals and their binding-protein is not clear. Recently, it was suggested by genetic data that VirA recognizes phenolic compounds directly (13). However, it was reported that deletions of the periplasmic domain of VirA and/or its two membrane spanning portions have little effect on its ability to mediate *vir* gene induction by phenolics (14). These results imply that VirA senses the phenolic compounds either directly in the cytoplasmic domain, or indirectly through another receptor protein.

To identify the inducer-binding protein, the synthesis of radiolabeled acetosyringone (AS) was pursued and we report here how a radiolabeled AS of high specific activity was prepared.

## EXPERIMENTAL

**General.** –Tritium gas (97.9 % $^3H_2$) was purchased from Oak Ridge National Laboratories and all other chemicals were obtained from Aldrich Chemical Co. Tritiated samples were counted in Opti-Fluor scintillant (Packard) with a Packard 1500 Tri-Carb liquid scintillation counter. $^1$H-NMR spectra ($CDCl_3$ or $C_6D_6$) and $^3$H-NMR spectra ($C_6D_6$) were recorded on an IBM-Bruker AF-300 NMR spectrometer. Mass spectral data were obtained on a Hewlett-Packard 5790A gas chromatograph with a 5970A mass selective detector. Reactions were monitored by TLC on precoated silicagel $60F_{254}$ plates.

***3',5'-Dimethoxy-4'-isopropoxyacetophenone* (2).**

To a mixture of acetosyringone **1** (5.0 g, 25.5 mmol) and $K_2CO_3$ (7.5 g, 54.3 mmol) in dry DMF (30 mL) was added isopropyl bromide (10.0 g, 81.3 mmol). The mixture was heated at reflux under Ar. After 4.5 h, the mixture was cooled, poured into water (50 mL), and extracted with chloroform (2 x 50 mL). The organic extracts were combined, washed with brine (2 x 20 mL), and dried over $Na_2SO_4$. The filtrate solution was concentrated *in vacuo* and the residue was washed with hexane : EtOAc (1 : 1) to yield 4.0 g of product (65.9 %): $R_f$ = 0.58 in hexane : EtOAc (1 : 1); $^1$H-NMR (300 MHz, $CDCl_3$) δ 1.27 (d, J=6.2 Hz, 6H), 2.54 (s, 3H), 3.86 (s, 6H), 4.46 (m, 1H), 7.18 (s, 2H); MS, m/z (rel..intensity) 238 ($M^+$, 0.2), 196 (43), 181(100).

***3',5'-Dihydroxy-4'-isopropoxyacetophenone* (4).**

To a solution of 3',5'-dimethoxy-4'-isopropoxyacetophenone **2** (2.0 g, 8.4 mmol) and $CH(OCH_3)_3$ (20 mL, 182.8 mmol) was added 0.5 g of Amberlyst-15. The mixture was stirred at RT under Ar. After 30 hr, the catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The product was purified on silica gel with hexane : EtOAc (2 : 1) to give 1.14 g of ketal **3** (47.9 %): $R_f$ = 0.77 in hexane : EtOAc (1 : 1); $^1$H-NMR

(300 MHz, $CDCl_3$) δ 1.20 (d, J=6.2 Hz, 6H), 1.49 (s, 3H), 3.15 (s, 6H), 3.79 (s, 6H), 4.29 (m, 1H), 6.67 (s, 2H); MS, m/z (rel. intensity) 284.2 ($M^+$,12), 211 (100), 181 (9), 89 (2). To a solution of $Ph_2PH$ (1.4 mL, 8.18 mmol) in dry THF (50 mL) cooled in an ice bath was added 5.2 mL of n-BuLi (1.6 M in THF, 8.32 mmol). To the deep orange solution, ketal **3** (0.93 g, 3.27 mmol) in dry THF (2 mL) was added dropwise. The solution was heated at reflux under Ar. After 3 hr, the solution was cooled and mixed with ether (50 mL) and water (50 mL). The organic layer was separated out and the aqueous layer was washed with ether (2 x 40 mL) to remove $Ph_2PCH_3$ and $Ph_2PH$. The aqueous layer was acidified with 1N HCl and then extracted with ether (3 x 30 mL). The extracts were washed with brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. The residue was chromatographed on a silica column with hexane : EtOAc (1 : 1) to purify the demethylated product **4** (0.55 g, 80.0 %): $R_f$ = 0.35 in hexane : EtOAc (1 : 1); $^1$H-NMR (300 MHz, $CDCl_3$) δ 1.34 (d, J=6.1 Hz, 6H), 2.52 (s, 3H), 4.68 (m, 1H), 5.96 (s, 2H), 7.17 (s, 2H); MS, m/z (rel. intensity) 210 ($M^+$, 6), 168 (29), 153 (100).

***3',5'-[$^3H_3$]Dimethoxy-4'-hydroxyacetophenone* (6).**

Two different methods were used to generate tritiated methyl iodide. The $C^3H_3I$ (6 Ci) generated by method 1 (Scheme 2) was trapped in a mixture of 3',5'-dihydroxy-4'-isopropoxyacetophenone **4** (10 mg, 0.047 mmol) and $K_2CO_3$ (40 mg, 0.29 mmol) in DMF (0.5 mL) at -80 °C. In the case of method 2 (Scheme 2), the reaction was carried out with 5 mg of **4** (0.024 mmol) and 5 Ci of tritiated methyl iodide. The mixture was stirred at 4 °C for 30 min and then at RT for 1.5 hr. After removal of unreacted $C^3H_3I$ and half of the DMF under reduced pressure, the reaction mixture was treated with EtOAc (3 mL) and washed with water (2 x 3 mL) and brine (2 mL). The organic layer was dried over $Na_2SO_4$, filtered, and concentrated to dryness in a nitrogen stream to give 3',5'-[$^3H_3$]dimethoxy-4'-isopropoxyacetophenone **5**: 1.3 Ci (21.6 %) by method 1 and 1.7 Ci (34.0 %) by method 2; $R_f$ = 0.39 in hexane : EtOAc (2 : 1). The product **5** was dissolved in dry benzene (I mL), the solution cooled in an ice-water bath and treated with $TiCl_4$ (0.1 mL, 1M solution in benzene). The solution was stirred at RT for 15 min and treated with more benzene (3 mL). The organic solution was washed with water (2 x 2 mL) and brine (1 mL), dried over $Na_2SO_4$, filtered, and concentrated in a nitrogen stream to give 3',5'-[$^3H_3$]dimethoxy-4'-hydroxyacetophenone **6**: 0.97 Ci (76.9

% from **5**) by method 1 and 1 Ci (58.8% from **5**) by method 2; $R_f$ = 0.21 in hexane : EtOAc (2 : 1); $^3$H-NMR (320 MHz, $C_6D_6$), δ 3.18($C^3H_3$), 3.21($CH^3H_2$), 3.24($CH_2{}^3H$) (Figures 1 and 2).

***Generation of $C^3H_3I$*** :

**Scheme 2, method 1** –The tritiated methyl iodide was prepared by the method of Saljoughian *et al.* (18) from 4-[methyl-$^3$H]methoxy biphenyl (**8**, 6 Ci, expected specific radioactivity = 60 - 90 Ci/mmole) with glacial acetic acid (1 mL) and HI (57 %, 200 μL) for the methylation of 3',5'-dihydroxy-4'-isopropoxyacetophenone **4** (10 mg, 0.047 mmol).

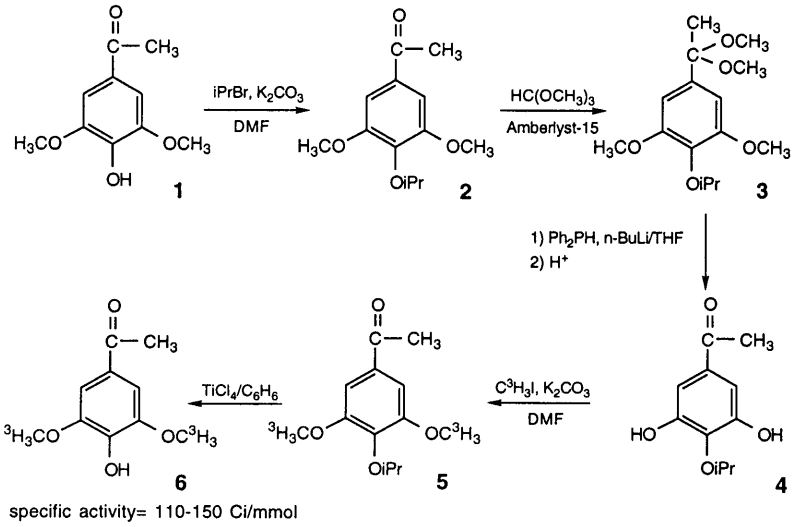
**Scheme 2, method 2** –The tritiated methyl iodide was prepared by the reduction of $CO_2$ with $LiAl^3H_4$. The $LiAl^3H_4$ was prepared by the method of Andres et al. (19) on a 0.25 mmole scale in a manifold consisting of two bulbs isolated by Teflon stopcocks, one connected to a small condenser for generation of methyl iodide and another for methylation. To the prepared $LiAl^3H_4$ in THF (500 μL) was injected 2.5 mL of $CO_2$ gas from a gas syringe, and the solution was stirred for 1 hr to generate $C^3H_3OH$ (**9**). After THF was removed, 2(3)-(tetrahydrofurfuryloxy)tetrahydropyran (TFTP, 100 μL) and HI (57 %, 500 μL) were added into the reaction bulb. The bulb was heated at 155 °C and stirred for 30 min to generate $C^3H_3I$. The $C^3H_3I$ (5 Ci, expected specific radioactivity = 60 - 90 Ci/mmole) was transferred (maintaining the temperature of the generation bulb at 155 °C) through a condenser maintained at 48 °C (bp of $CH_3I$) with water to the methylation bulb containing a precooled (ice-bath) mixture of 3',5'-dihydroxy-4'-isopropoxyacetophenone **4** (5 mg, 0.024 mmol), DMF (300 μL) and $K_2CO_3$ (20 mg, 0.14 mmol).

## RESULTS AND DISCUSSION

Acetosyringone is one of the inducers involved in *A. tumefaciens* - plant interactions. The reason for selecting AS as a model inducer is that AS is the most prominent inducer found in a variety of plants and its structure is sufficient to understand the induction mechanism. Deprotonation of the *p*-hydroxyl functional group in AS by the binding-protein and then electron flow through the acetophenyl backbone are

presumed to initiate the induction process (15). The two meta-oriented methoxy groups are speculated to augment the affinity to the binding protein, although it was reported recently that the *vir* genes of *A. tumefaciens* KU12 strain were induced little by acetosyringone but induced well by 4-hydroxyacetophenone which lacks methoxy groups (13). In order to identify the AS binding protein, tritium-labeled AS ($^3$H-AS) was prepared with a high specific radioactivity as shown in Scheme I.




**Scheme 1**. Synthesis of 3,5-[$^3H_6$]dimethoxy-4-hydroxyacetophenone ($^3$H-AS).

The use of $^3$H (or $^{14}$C) to search for receptors has the advantage of very high sensitivity and that the compound can be left chemically unaltered, and thus biologically indistinguishable from the unlabeled compound. Two-key steps in the synthesis of $^3$H-AS were the selective demethylation of the methoxy groups in the presence of the isopropoxy group using diphenylphosphine ($Ph_2PH$) with n-BuLi (16) and the use of $TiCl_4$ to selectively cleave the isopropoxy group in the presence of the tritiated methoxy groups (17). The reagent $Ph_2P^-$, generated from $Ph_2PH$ and n-BuLi, acts via $S_N2$ displacement which favors attack at the less sterically hindered methyl groups rather than the isopropyl group to produce 3',5'-dihydroxy-4'-isopropoxyacetophenone **4**. After methylation of **4** with $C^3H_3I$, the final product **6**, 3',5'-[$^3H_3$]dimethoxy-4'-hydroxyacetophenone, was obtained by reacting 3',5'-[$^3H_3$]dimethoxy-4'-isopropoxyacetophenone **5** with $TiCl_4$, a mild Lewis acid cleaving

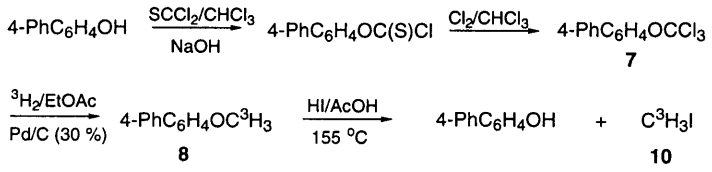
preferentially at the isopropyl group of which resulting cationic species is more stable than that of the methyl groups.

The methylating agent, tritiomethyl iodide **10** was generated by two different methods, shown in Scheme 2. The tritiomethyl iodide in method 1 was made by dehalogenation of the chlorinated precursor **7** using tritium gas with Pd/C and subsequent cleavage of the methyl ether **8** with HI. The chemical yield from the dehalogenation reaction is about 30 % calculated from experiments using deuterium, and the maximum percentage of $CH_3$ speies is about 3 % (18). In our experiment, the tritium abundance for the three methyl species in the product **6** was measured by tritium NMR spectroscopy (Figure 1). The specific radioactivity of the methyl iodide and other intermediate methyl species may be inferred from the specific radioactivity of the final product methyl groups. With measured tritium NMR peak intensity ratios ($C^3H_3$ : $C^3H_2H$ : $C^3HH_2$ = 44 : 42 : 14), assuming the specific radioactivity of tritium as 28.76 Ci/milliatom and the maximum abundance of $CH_3$ as 3 % (18), we calculate a specific radioactivity of 56.1 Ci/mmole for these methyl groups. Due to exchange of tritium with hydrogen by exchangeable protons on the catalyst, glassware, reagent or solvent during the reductive dehalogenation, the specific radioactivity was substantially lower than the theoretical maximum specific radioactivity of 86.28 Ci/mmole. However, this method has the advantage that the chlorinated precursor **7** can be stored until it is required.

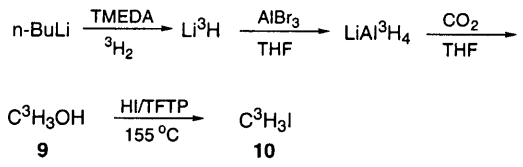
The preparation of tritiomethyl iodide **10** by method 2 was similar to the literature (21) where $C^3H_3OH$ (**9**) was used as the tritiomethyl precursor. The specific radioactivity of $^3H$-AS produced by this method was higher than that from method 1. The abundance of $C^3H_3$ species was much higher for this method, as shown by the $^3H$-NMR spectrum of the final product **6** (Figure 2). With measured NMR peak intensity ratios ($C^3H_3$ : $C^3H_2H$ : $C^3HH_2$ = 87 : 7 : 6) the calculated specific radioactivity of methyl group was 72.6 Ci/mmole. The key reagent for generating $C^3H_3OH$ from $CO_2$, $LiAl^3H_4$, was freshly prepared from $Li^3H$ which was made by reacting n-BuLi with $^3H_2$ gas (19). The $Li^3H$ was converted to $LiAl^3H_4$ by $AlBr_3$. This method to produce $C^3H_3OH$ from $CO_2$ and $LiAl^3H_4$ is convenient and mild compared to the previous synthesis of $C^3H_3OH$ from $CO_2$ and $^3H_2$ under high pressure and high temperature conditions (20).

**method 1.**




**method 2.**




**Scheme 2.** Preparations of $C^3H_3I$.

Based on the specific radioactivity of the tritiomethyl iodide generated by methods 1 and 2, 56.1 and 72.6 Ci/mmole, respectively, the values for $^3$H-AS containing two tritiated methyl groups are 112.2 and 145.2 Ci/mmole, respectively. This high specific radioactivity will be sufficient to identify the AS receptor protein at the picomole level.

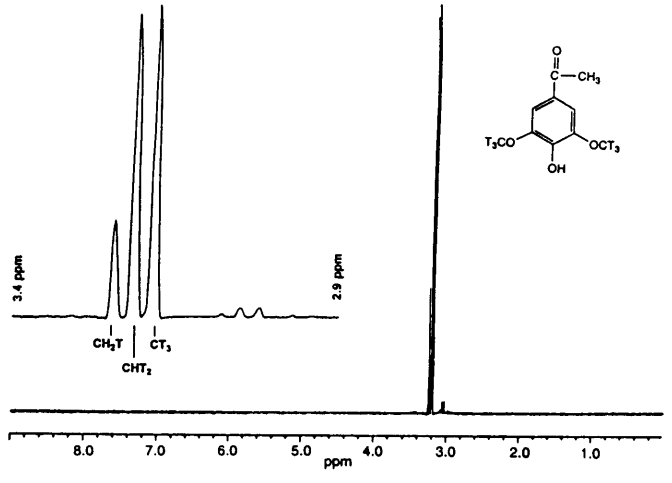



**Figure 1.** $^3$H-NMR spectrum of $^3$H-AS prepared from $C^3H_3I$ generated by method 1.

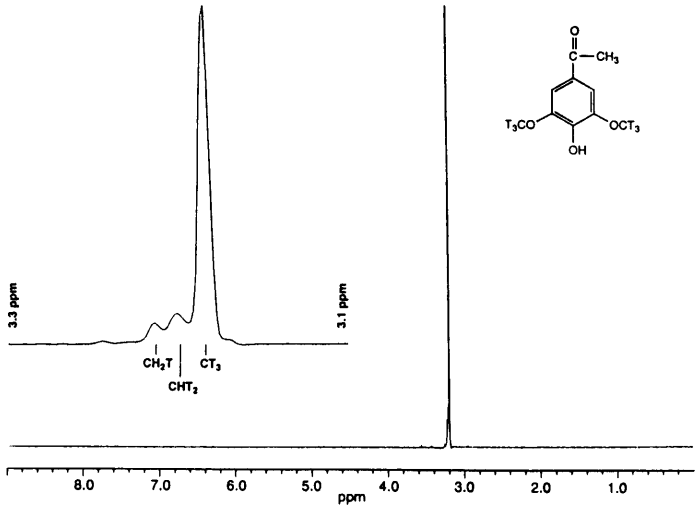



**Figure 2.** $^3H$-NMR spectrum of $^3H$-AS prepared from $C^3H_3I$ generated by method 2.

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