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IDENTIFICATION OF NAA-L-ASPARTATE AMIDE AS THE MAJOR METABOLITE SYNTHESIZED BY TOBACCO MESOPHYLL PROTOPLASTS INCUBATED IN THE PRESENCE OF THE AUXIN ANALOGUE NAA

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Key Word Index—*Nicotiana tabacum*, Solanaceae, protoplast, NAA; auxin-amino acid; conjugates; isolation; identification

Abstract—A major metabolite of naphthalene 1-acetic acid (NAA) is rapidly accumulated by tobacco mesophyll protoplasts induced to divide by this growth regulator. A comparison of the natural product with various chemically synthesized NAA-amino acid conjugates was performed. The metabolite was identified as NAA-aspartate amide by negative CIMS. The biological activity of NAA-aspartate on protoplasts was further studied. The significance of the accumulation of this metabolite in dividing protoplasts is discussed.

INTRODUCTION

The physiology of plant protoplast division is still far from understood. Whilst protoplasts from a limited number of plant species (*Nicotiana*, *Datura*, *Petunia* . . .) are able to divide those from other species are resistant to the induction of proliferation by various growth regulators. A comparison of the metabolism of growth regulator in dividing and non-dividing protoplasts should help to explain this difference. Tobacco mesophyll protoplasts can be reproducibly induced to divide with a high efficiency [1]. Division is stringently dependent of the presence of auxin and cytokinin analogues such as naphthalene acetic acid (NAA) and benzyladenine in the culture medium.

NAA is very rapidly taken up by cells (60–70% of the initial amount of NAA in the culture medium is taken up by protoplast cultures during the first day of incubation) and further metabolized [1]. The purpose of this work was to characterize the major metabolite being synthesized and accumulated in protoplasts, to prepare large amounts of the molecule and to study its eventual growth stimulating properties on protoplast preparations.

RESULTS AND DISCUSSION

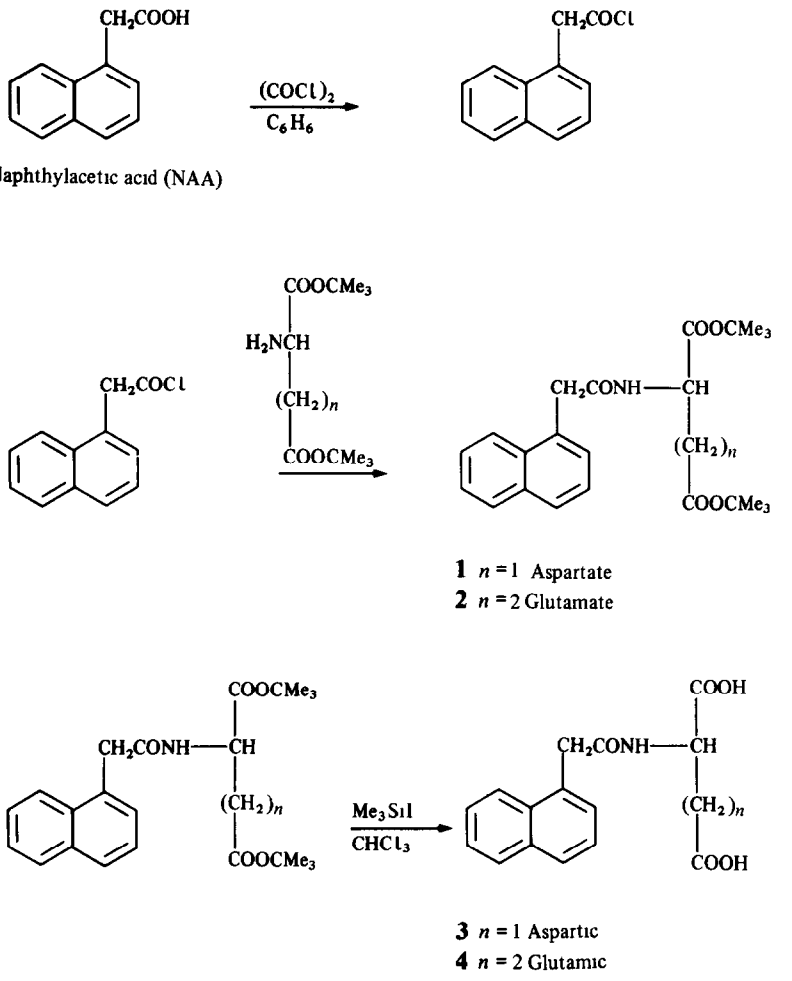
Three metabolites of NAA were detected in extracts of protoplast incubated in the presence of [^{14}C]NAA. The major metabolite (R_f 0.40) accounted for approximately 50% of the NAA initially supplied to the cells [1]. This metabolite was ether extractable from acidic aqueous solutions and was therefore thought to be an amino acid conjugate of NAA since sugar conjugates are generally not ether extractable [2] and aspartate conjugates of IAA and NAA had been detected in different tissues of plants incubated in the presence of these auxins [3, 4].

However, on negative CIMS analysis, the major metabolite gave a diagnostic peak at *m/z* 255 which was inconsistent with it being an amino acid conjugate. Thus NAA-alanine amide would be expected to give a molecular peak at *m/z* 257 and NAA-aspartic amide would give a peak at *m/z* 301. A structure of the type NAA-putrescine monoamide would theoretically give a value of 256 M^- or 255 $[M-H]^-$. This last hypothesis was attractive since the involvement of phenylamines in differentiation processes has been described in several instances [5]. These various molecules were therefore synthesized. The structures corresponding to the putrescine and alanine amide were rapidly eliminated without ambiguity on the basis of their R_f values on TLC.

The L-aspartic acid or L-glutamic acid conjugates of NAA were synthesized (Scheme 1) in order to do a detailed comparison of these molecules to the natural product extracted from protoplasts. The NAA amides were synthesized by reaction between the acid chloride and the *t*-butylesters of the amino acids in the presence of triethylamine. The diacidamides **3** and **4** were obtained by the action of trimethylsilyliodide on the *t*-butylesters **1** and **2** in chloroform solution [7, 8].

The physical data of the *t*-butylesters intermediates and of the final conjugates are given in the Experimental. The mass spectra of the diacid amides **3** and **4** were performed by Negative Chemical Ionisation [9, 10]. The spectra were sometimes characterized by weak quasi molecular ions at *m/z* 301 and 315 respectively, the intensity of these ions depending upon the experimental conditions. The most prominent peaks were at *m/z* 255 and 269 respectively which corresponded to $[301-(CO_2+H_2)]^-$ and $[315-(CO_2+H_2)]^-$ ions where 2H represents the acidic protons. Monoacids like NAA and NAA-alanine were not characterized by these diagnostic ions. Their spectra showed intense peaks at *m/z* 185 and 256 respectively, representative of $[M-H]^-$ ions.

We conclude that NAA-aspartate is indeed the major metabolite derived from NAA in protoplast cultures on the basis of the identity of mass spectra of the natural and synthetic product. This is further confirmed by their identical TLC properties and by an analysis of the amino



Scheme 1. Synthesis of NAA-L-aspartate and L-glutamate amides.

acid content of the natural product after acid hydrolysis (results not shown). The biological activity of synthetic NAA-aspartate amide was further studied. Concentrations as high as 150 μM were unable to induce the division of mesophyll protoplasts when the conjugate replaced NAA in the medium. These observations therefore suggest that NAA-aspartate amide is not biologically active and cannot substitute for the free auxin, most probably because the amide bond is not rapidly cleaved in protoplast cultures. The biosynthesis of the amide in protoplast therefore appears as a process of inactivation of the auxin supplied to cells rather than a mediator of auxin action.

EXPERIMENTAL

Plant material. Tobacco mesophyll protoplasts (*N. tabacum* cv Xanthi) were prepared from haploid plants as described previously [6] After overnight digestion by cellulase, protoplasts were washed twice and incubated in medium T_0 at a density of 7 $\times 10^4$ p/ml. Protoplasts were incubated generally in the presence of 15 μM NAA and 5 μM benzyladenine First mitoses were observed 3 days after plating. Plating efficiencies were generally higher than 80%

Extraction of NAA metabolites from protoplast derived cells. The metabolites of NAA were identified by TLC of extracts from cells labelled with [^{14}C]NAA [1]. The purification of large amounts of these metabolites was performed from 10^8 protoplasts incubated for 4 days in the presence of 15 μM NAA Cells were washed ($\times$ 2) with saline osmoticum, and cell pellets were then extracted twice with 90% EtOH. The extracts were concd under vacuum and subjected to TLC on silica gel HF 254 developed with $CHCl_3$–EtOAc–HCO_2H (5.4 1). Fluorescent spots were eluted with 90% EtOH and used for further analysis.

Physical methods Mass spectra were recorded in a VG ZAB.2F mass spectrometer with a high pressure source. Ionisation by negative charges OH^- was made using a mixture of N_2O–CH_4 in a ratio 1 10 to produce under 1 torr pressure the reagent ions OH^- (filament current = 0.2 mA, ions acceleration = 8 kV and source temperature = 160°) [11].

Synthesis of intermediates and amides The amino acids, the 1-naphthylacetic acid and the different reagents used in this work were purchased from commercial sources and were used without purification. (a) *Intermediates.* 4 $\times$ 10^2 moles NAA (7 44 g) were dissolved with stirring under N_2 in 50 ml dry C_6H_6 and 25 ml dry Et_2O in a vessel cooled by an ice bath Two drops pyridine were added to the soln, then 3.45 ml oxalylchloride diluted in 50 ml dry benzene were added dropwise over a period of 1 hr with rapid stirring The soln was left overnight to warm to room temp with continuous stirring. An IR spectrum showed the disappearance

of NAA absorptions 3200 and 1710 cm^{-1} The acid chloride was characterized by carbonyl absorption at 1795 cm^{-1} ($CHCl_3$ soln). (b) *Synthesis of amides* t-*butylesters* **1** *and* **2**. The synthesis of NAA-L-aspartate *t*-butylester amide is described as an example In a vessel cooled in an ice bath, 1.84×10^{-2} moles of L-aspartate *t*-butylester (4.50 g) was dissolved into 51 ml dry Et_2O and 4.20 ml dry Et_3N. Under N_2 the above soln of acid chloride was reduced *in vacuo* to half vol and then added dropwise with continuous stirring to the aspartate *t*-butylester soln under N_2 After 3 hr, the mixture was allowed to warm to room temp After neutralization of the soln by solid $NaHCO_3$, followed by filtration, the mixture was washed (× 2), dried over K_2CO_3 and the solvents removed *in vacuo*. The yellow oil residue was chromatographed on silica gel H and eluted with increasing amounts of Et_2O in hexane The colourless oil isolated showed only a single spot on TLC in hexane–Et_2O (2.3) after irradiation with a UV lamp at 254 nm Yield after chromatography, 85–90%. (c) *Diacid amides* **3** *and* **4**. Among the methods we have tried, the best one is that described by Jung and Olah [7, 8] in $CHCl_3$ soln Other methods, like acidic methods in various solvents do not give good results or poor yields of impure materials

In a vessel cooled in an ice bath, 1.315 g ANA glutamate amide *t*-butylester was dissolved in 25 ml $CHCl_3$ (distilled over P_2O_5) Under N_2, 1.25 ml of Me_3SiI was added dropwise from a syringe with rapid magnetic stirring. The flask was kept at 0° for 24 hr In the case of aspartate amide the reaction was carried out at 0° and completed after 30 min. The reaction was worked up by quenching with a chilled 5% NaOH soln (50 ml) and extracting the aq soln (× 2) with 25 ml CH_2Cl_2. The basic aq. soln was made acidic by adding cautiously a chilled 10% HCl soln until the pH = 3 to 2. In most cases, the diacid was precipitated The isolated crystalline cake was washed with H_2O until neutral then with cold $CHCl_3$. The yield (crystallized white material) was 73–85%. The diacids were crystallized laboriously from EtOH or a mixture of C_6H_6–MeOH–$CHCl_3$ (× 3) before use Mps. compound **3**, 170–172° compound **4**, 188–191°. (d) *Spectroscopic and physical data of compounds* **1–4**. The IR spectra (neat) of the amide *t*-butylesters were characterized by absorption bands at 3300, 1730, 1665, 1600 and 1100 cm^{-1} ^1H NMR (60 MHz, $CDCl_3$, TMS as int. standard): NAA-L-aspartate amide **1**: δ 7.1–7.95 (7H, *m*, naphthyl ring), 6.37 (1H, *d*, NH–CH, J = 8 Hz), 4.63 (1H, *dt*, NH–CH, J = 8 Hz and 4 Hz), 4.00 (2H, *s*, naphthyl CH_2), 2.65 (2H, *t*, CH–CH_2, J = 4 Hz), 1.30 and 1.17 (9H and 9H, 2*s*, $2COOCMe_3$) NAA-L-glutamate amide **2**: δ 7.35–8.05 (7H, *m*, naphthyl ring), 6.03 (1H, *d*, NH–CH, J = 8 Hz), 4.50 (1H, *m*, NH–CH), 4.03 (2H, *s*, naphthyl CH_2), 2.00 and 1.80 (4H, *m*, CH_2CH_2), 1.35 and 1.10 (9H and 9H, 2*s*, 2 $COOCMe_3$). ^{13}C NMR (20 MHz, Fourier transform mode, $CDCl_3$, TMS as int. standard): **1**: δ 170.4, 169.6, 169.5 (each C=O), 134.0–123.8 (naphthyl ring), 82 (–CH.COO.CMe_3, 81.3 (–CH_2 COO CMe_3), 49.2 (NH–CH), 41.5 (naphthyl-CH_2), 37.4 (–CH–CH_2–) and 27.8 (–CMe_3); **2**: δ 171.9, 170.6, 170.5 (each C=O), 134.0–123.8 (naphthyl ring), 82.1 (–CHCOO–CMe_3), 80.5 (–CH_2COO–CMe_3), 52.2 (NH–CH), 41.6 (naphthyl-CH_2), 27.6 (–CH–CH_2–), 31.2 (–$CHCH_2CH_2$–C), 28.0 and 27.8 (–CMe_3). ORD values

$$\mathbf{1}\,[\alpha]^{\lambda}_{OR} = \frac{\begin{matrix}589 & 578 & 546 & 436\,nm\end{matrix}}{\begin{matrix}+42.6 & +44.4 & +50.6 & +87.5\end{matrix}} \quad (c\ 2.435,\ CHCl_3)$$

The corresponding values for **2** (*c* 2.816, $CHCl_3$), **3** (*c* 1.680, 95% EtOH) and **4** (*c* 2.095, 95% EtOH) were −6.9, −7.2, −8.2, −14.7, +10.4, +10.8, +12.1, +18.1; −7.3, −7.6, −8.6, −14.8

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