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Stable-Isotope Labeled Metabolites of the Phytohormone, Indole-3-Acetic Acid

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SUMMARY

1,3-Dicyclohexylcarbodiimide-mediated condensation of [3a,4,5,6,7,7a-$^{13}C_6$]indole-3-acetic acid with the bis(*tert*-butyl) esters of L-aspartic or L-glutamic acids, followed by removal of the ester groups by dilute alkali, afforded *N*-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-aspartic and *N*-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-glutamic acids, labeled forms of compounds involved in the regulation of plant growth and development. The corresponding conjugates of (*R,S*)-2,3-dihydro-2-oxoindole-3-acetic acid, which are likewise of physiological significance, were labeled with ^{15}N in the amino acid moieties and were synthesized *via* the *N*-hydroxysuccinimide ester.

KEY WORDS: plant hormone, auxin, amino acid conjugate, stable isotope labeling, indole-3-acetic acid, 2,3-dihydro-2-oxoindole-3-acetic acid

INTRODUCTION

The phytohormone (auxin), indole-3-acetic acid (**1**), is metabolized to a variety of conjugates of outstanding importance in the regulation of plant growth and development (1). To elucidate their functions in detail, isotope-labeled standards are needed to be used, for instance, in turn-over studies and in quantitative analyses by methods based on isotope-dilution. A number of radioactive conjugates of **1** have already been prepared (*e. g.* 2 - 6), but little attention has been devoted to labeling with stable isotopes (7), although this approach has at least two major advantages: *1)* lower detection limits when coupled with sample analysis by mass spectroscopy and *2)* greater reliability as there is no spontaneous radiolysis which can be a serious problem with radioactive indoles (8, 9). We here focus on labeled analogues (Figure 1) of the endogenous conjugates (**5**, **7**) of **1** with aspartic and glutamic acids (10 - 13), and of the corresponding conjugates (**6**, **8**) of (*R,S*)-2,3-dihydro-2-oxoindole-3-acetic acid (**2**) which likewise are known

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(**6**), or suspected (**8**), plant metabolites (14 - 16). To make optimal use of commercially available starting materials, conjugates **5** and **7** were labeled with ^{13}C in the indole ring, and conjugates **6** and **8** with ^{15}N in the amino acid moieties.

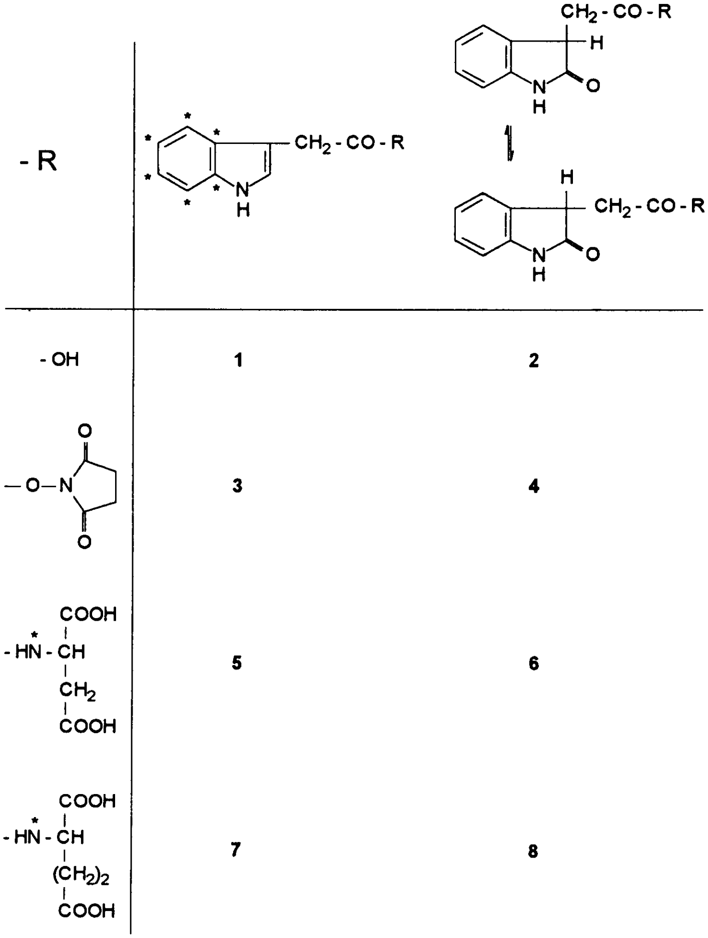


Fig. 1. Summary of the compounds prepared. L-Amino acid residues are implied unless explicitly stated otherwise. The presence of heavy isotopes, at the positions indicated above by an asterisk, is specified in the text by a suffix (in square brackets) preceding the compound number, *e. g.* [^{13}C$_6$]**5** or [^{15}N]**6**.

EXPERIMENTAL

General.

Analytical grade chemicals and solvents were purchased from commercial sources. In particular, [3a,4,5,6,7,7a-^{13}C$_6$]indole-3-acetic acid ([^{13}C$_6$]**1**), [^{15}N]-L-aspartic acid and [^{15}N]-L-glutamic acid were from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. *N*-(indol-3-ylacetoxy)succinimide was synthesized as described (19, 20). (*R,S*)-2,3-Dihydro-2-oxoindole-3-acetic acid (**2**) was prepared by the method of Hinman and Bauman (21). For optimal yields, the reaction was performed in 95% (by vol.) *tert*-butanol (Merck).

Solutions were concentrated *in vacuo*, at 25 - 30° C, using a rotary evaporator. Melting points (uncorrected) were determined in open capillaries. Thin-layer chromatography was on glass plates coated with silica gel 60 F_{254} (Merck) using the following solvent systems: A, dichloromethane/methanol/acetic acid (90:10:1; by vol.); B, *n*-butanol/acetic acid/water (80:3:17; by vol.); C, chloroform/methanol/water (85:14:1; by vol.) and D, 2-propanol/ethyl acetate/NH_4OH (35:45:20; by vol.). Solvents A and B were utilized to monitor the purification of amino acid conjugates **5** - **8** and their labeled counterparts; solvents C and D were employed as specified below. Spots on chromatograms were detected by uv fluorescence (excitation at 254 nm) and, for indoles, by Ehmann's reagent (17). Column chromatography was on Sephadex LH-20 (77 x 2.5 cm i. d.) eluted with 2-propanol/water (1:1; by vol.). Preparative HPLC was on polygosil C18, particle size 5 - 60 μm (250 x 10 mm i. d.), eluted at a flow rate of 2 ml/min, with a solvent containing increasing levels of methanol (linear gradient from 10% to 80% by vol. in 30 min; hold final concentration for 15 additional min) in 1% aqueous acetic acid. Analytical HPLC was on a column (100 x 4.6 mm i. d.) of Spheri-5 RP18, particle size 5 μm, eluted at 1 ml/min with the solvent systems specified below for each individual separation. The effluent from all the above columns was monitored for absorbance at 280 nm (for indoles) or 226 nm (for 2,3-dihydro-2-oxoindoles). The structures of the prepared compounds were verified by combined high pressure liquid chromatography-mass spectrometry with ionization by fast-atom bombardment (FAB HPLC/MS), as described previously (18). In brief, the sample was passed through a 250 x 0.32 mm i. d. capillary HPLC column packed with C_{18}-reversed phase silica gel (LC packings, Amsterdam, The Netherlands), particle size 5 μm, and eluted (4 μl/min) with 30% (by vol.) aqueous methanol containing 1% (by vol.) each of acetic acid and glycerol (matrix for FAB). The effluent was introduced, *via* a frit-FAB HPLC/MS interface, into the ion source (kept at 50° C) of a double focusing JEOL JMS SX 102 mass spectrometer. Ions were generated with a beam of 5 kV xenon atoms at an emission current of 20 mA, and positive-ion mass spectra were acquired. For exact mass measurements, polyethylene glycol (PEG 300) was added to the HPLC solvent (0.05%; by vol.) to furnish the referent ions, and the resolution was set to R = 5000. Also, compounds [$^{13}C_6$]**5**, [^{15}N]**6**, [$^{13}C_6$]**7**, and [^{15}N]**8** were converted to their dimethyl esters (using diazomethane) to reduce peak widths in capillary HPLC, and to enhance sensitivity in FAB MS. Notably, the ion subjected to exact mass determination was the protonized molecular ion $[M + H]^+$.

N-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-aspartic acid ([$^{13}C_6$]5).

1,3-Dicyclohexylcarbodiimide (32.5 mg, 158 μmol) was stirred into a solution of [$^{13}C_6$]**1** (9.0 mg, 50 μmol), L-aspartic acid bis(*tert*-butyl) ester hydrochloride (36.2 mg, 128 μmol), and tri-*n*-butylamine (29 μl, 22.6. mg, 122 μmol) in acetonitrile (5 ml) precooled to -10° C. After a further 30 min of stirring at that temperature, the mixture was kept at 3 - 5° C until the condensation was about 95% complete (18 h), as verified by TLC (solvent C). The 1,3-dicyclohexylurea formed was filtered off, and the filtrate was concentrated nearly to dryness. The residue was dissolved in a mixture of 2 N NaOH (2 ml) and ethanol (3 ml), and kept at 55° C until TLC (solvent D) indicated complete saponification of both *tert*-butyl ester groups (8 h). The mixture was then extracted with diethyl ether (4 x 5 ml) to remove neutral impurities. The aqueous phase was acidified (pH 1 - 2) and partitioned against *n*-butanol (5 x 5 ml) The residue obtained by evaporation of the organic phase was further purified by Sephadex chromatography and preparative HPLC (retention time: 24 min) as detailed above. The pure title compound (overall yield 15%) was characterized by its mass spectrum (Figure 2). That of the dimethyl ester showed a parent peak ($[M + H]^+$) at *m/z* 325.151. [$^{13}C_6$]$C_{10}H_{19}N_2O_5$ requires 325.150.

N-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-glutamic acid ([$^{13}C_6$]7).

[$^{13}C_6$]**1** (9.4 mg, 52 μmol), L-glutamic acid bis(*tert*-butyl) ester hydrochloride (39 mg, 132 μmol), and tri-*n*-butylamine (24.5 mg, 31.5 μl, 132 μmol) in acetonitrile (5 ml) were reacted with 1,3-dicyclohexylcarbodiimide (32 mg, 155 μmol) as described for the preparation of [$^{13}C_6$]**5**. As the condensation was not complete after 24 h (TLC, solvent D), a further excess of the following reactants was added: L-glutamic acid bis(*tert*-butyl) ester hydrochloride (40 mg, 135 μmol), tri-*n*-butylamine (33 μl, 139 μmol), and 1,3-dicyclohexylcarbodiimide (35 mg, 170 μmol). After a further 24 h the mixture was filtered, diluted with diethyl ether (10 ml) and extracted, in sequence, with water (2 x 5 ml), 4% (by vol.) H_3PO_4 (4 x 6 ml), 5% (by wt.) $NaHCO_3$ (4 x 6 ml), and brine (2 x 1 ml). The organic phase was evaporated and the protected conjugate was extracted from the residue (salts and 1,3-dicyclohexylurea) with ethanol. After evaporation, the residue was treated with a mixture of 2 N NaOH (2 ml) and ethanol (3 ml), at 55° C, for 1.5 h, and left at room temperature overnight. Further work-up and purification by Sephadex chromatography and preparative HPLC (retention time: 25 min) was as described for [$^{13}C_6$]**5**. The mass spectrum of the pure title compound (final yield 10%) is shown in Figure 2. That of the dimethyl ester showed a parent peak ([M + H]$^+$) at *m/z* 339.165. [$^{13}C_6$]$C_{17}H_{21}N_2O_5$ requires 339.165.

N-(Indol-3-ylacetyl)-DL-glutamic acid (DL-7).

A solution of glutamic acid monohydrate (679 mg, 4.12 mmol) in 10% (by wt.) $NaHCO_3$ (10.7 ml) was added to a suspension of **3** (1 g, 3.68 mmol) in dioxane (16 ml), and the mixture was stirred overnight at room temperature. It was then partitioned against ethyl acetate (4 x 20 ml). The organic phase was back-extracted with 10% (by wt.) K_2CO_3 (2 x 5 ml) which was added to the aqueous phase of the original ethyl acetate partitioning. The combined aqueous phase was acidified to pH 2.5 and extracted with *n*-butanol (4 x 25 ml, readjusting the pH of the aqueous phase). The extract was washed with brine and evaporated. After complete removal of the *n*-butanol, by repeated coevaporation with water, the residue was crystallized from water to yield pink crystals (692 mg), and a second lot (77 mg) from the partially concentrated mother liquors (crude yield: 69%). The combined crystalline fractions were dissolved in 25% (by vol.) aqueous methanol (65 ml) and 5 equal aliquots of this solution were passed each through a reversed-phase cartridge (Varian Bond Elut C18, 1.5 x 1 cm, previously rinsed with methanol and equilibrated with 25% aqueous methanol) eluting with the same solvent. The DL-**7** recovered was recrystallized from water and twice from 20% (by vol.) aqueous ethanol to yield the pure title compound (523 mg, 47%) as white crystals, m. p. 182° C. Lit. (L-isomer): 162.5° C (22), 182 - 184° C (24). [^{13}C]-NMR data (methyl sulfoxide-d_6); indol-3-ylacetyl moiety: δ 124.0 (C-2), 109.1 (C-3), 127.6 (C-3a), 119.1 (C-4), 118.6 (C-5), 121.3 (C-6), 111.6 (C-7), 136.5 (C-7a), 32.5 (CH_2), 171.4 (CONH) ppm; glutamic acid moiety: δ 173.9 (C-1), 51.5 (C-2), 26.6 (C-3), 30.2 (C-4), 174.2 (C-5) ppm. [^{1}H]-NMR data (methyl sulfoxide-d_6); indol-3-ylacetyl moiety: δ 10.87 (broad s, 1 H, H-1), 7.20 (d, 1 H, $J_{1,2}$ = 2.1 Hz, H-2), 7.56 (d, 1 H, $J_{4,5}$ = 7.6 Hz, H-4), 6.96 (t, 1 H, $J_{5,6}$ = 7.4 Hz, H-5), 7.07 (t, 1 H, H-6), 7.34 (d, 1 H, $J_{6,7}$ = 8.1 Hz, H-7), 3.59 (d, 2 H, $J_{CH2,H\text{-}2}$ = 1.2 Hz, CH_2) ppm; glutamic acid moiety: δ 8.29 (d, 1 H, J = 8.0 Hz, NH), 4.22 (td, 1 H, CH-2), 1.98 (dtd, 1 H, $J_{vic\text{-}1}$ = 5.4 Hz, $J_{vic\text{-}2}$ = 8.0 Hz, J_{gem} = 13.8 Hz, CH_I -3), 1.79 (dtd, 1 H, $J_{vic\text{-}1}$ = 6.3 Hz, $J_{vic\text{-}2}$ = 8.5 Hz, CH_{II}-3), 2.28 (t, 2 H, CH_2-4), 12.4 (broad s, 2 H, 2 COOH) ppm. The mass spectrum of DL-**7** was essentially as shown in Figure 2 (right), except for an isotope shift (- 6 amu for all major ions) due the fact that the benzene part of the indole moiety was not ^{13}C enriched. The parent peak ([M + H]$^+$) was at *m/z* 305.117 (free DL-**7**). $C_{15}H_{17}N_2O_5$ requires 305.114.

N-((*R,S*)-2,3-Dihydro-2-oxoindol-3-ylacetoxy)succinimide (4).

To a stirred solution of **2** (352 mg, 1.84 mmol) and *N*-hydroxysuccinimide (234 mg, 2.03 mmol) in a mixture of dioxane (5 ml) and ethyl acetate (2.5 ml) 1,3-dicyclohexylcarbodiimide (418 mg, 2.03 mmol) was added, in 5 aliquots, through 30 min, at -8° C. After a further 1 h at +2° C, the 1,3-dicyclohexylurea was filtered off and the filtrate was concentrated to a yellowish half-solid. Recrystallization from dioxane afforded white crystals (278 mg, 52%), m. p. 185 - 187° C, FAB HPLC/MS: m/e 289 (100%) $[M + H]^+$, 174 (25%) $[M - OSu]^+$, 146 (75%) $[\text{2-hydroxyquinolinium}]^+$; 132, 133, 134 (5 - 10%) $[\text{dihydro-2-oxoindolium} + (0 - 2)\ H]^+$.

$[^{15}N]$*N*-((*R,S*)-2,3-dihydro-2-oxoindol-3-ylacetyl)-L-aspartic acid ($[^{15}N]$6).

A solution of $[^{15}N]$-L-aspartic acid (23.7 mg, 178 μmol) in 10% (by wt.) $NaHCO_3$ (1 ml) was added to a suspension of **4** (45.3 mg, 157 μmol) in dioxane (2 ml), and the mixture was stirred at room temperature, for 1.5 h. It was then extracted with ethyl acetate (2 x 5 ml). The aqueous phase was acidified to pH 2.5 and partitioned against *n*-butanol. The organic phase was washed with brine and concentrated. The residue was passed through the standard column of Sephadex LH-20 to yield 9.3 mg (19%) of the title compound. HPLC (8% methanol in 1% aqueous acetic acid): doublet at retention times 7.7 and 9.5 min. The mass spectrum is shown in Figure 3. That of the dimethyl ester showed a parent peak ($[M + H]^+$) at *m/z* 336.121. $[^{15}N]C_{16}H_{19}N_2O_6$ requires 336.121.

$[^{15}N]$*N*-((*R,S*)-2,3-dihydro-2-oxoindol-3-ylacetyl)-L-glutamic acid ($[^{15}N]$8).

Ester **4** (50 mg, 174 μmol) in dioxane (3 ml) and $[^{15}N]$-L-glutamic acid (39 mg, 264 μmol) in 10% (by wt.) $NaHCO_3$ (1.5 ml) were reacted, and the product was purified, as described for $[^{15}N]$**6**. Yield after Sephadex chromatography: 16 mg (29%). HPLC (10 % methanol in 1% aqueous acetic acid): doublet at retention times 8.7 and 10.6 min. The mass spectrum is presented in Figure 3. That of the dimethyl ester showed a parent peak ($[M + H]^+$) at *m/z* 350.138. $[^{15}N]C_{17}H_{21}N_2O_6$ requires 350.137.

RESULTS AND DISCUSSION

The compounds prepared are summarized in Figure 1. Conjugates $[^{13}C_6]$**5** and $[^{13}C_6]$**7** were synthesized by dicyclohexylcarbodiimide-mediated condensation of $[^{13}C_6]$**1** with the bis(*tert*-butyl) esters of L-aspartic and L-glutamic acids, followed by saponification of the ester groups under conditions minimally affecting the newly formed peptide bond. Such deprotection with dilute alkali was preferred to the more common use of trifluoroacetic acid because the indole ring is acid sensitive. The synthetic procedure has already been used (5) to make submicrogram amounts of radiolabeled *N*-($[2\text{-}^{14}C]$indol-3-ylacetyl)-L-aspartic acid. Modifications in accordance with the different reaction dynamics when working on a fifty-micromol scale include: *1)* addition of an equimolar amount of tri-*n*-butylamine to neutralize the HCl liberated when the commercial amino acid bis(*tert*-butyl) ester hydrochlorides undergo *N*-substitution, *2)* longer reaction times, *3)* removal of the ester groups by ethanolic, rather than by aqueous, NaOH (in which the esters are insoluble), and *4)* more complex purification procedures for intermediates and final products. While the synthesis of $[^{13}C_6]$**5** proceeded smoothly, the condensation of $[^{13}C_6]$**1** with L-glutamic acid bis(*tert*-butyl) ester required the addition of a second aliquot of the unlabeled reactants to proceed to ca. 90%

completion (estimated by TLC, solvent D). Also, TLC (solvent D) indicated minor formation of $[^{13}C_6]$**1** during the saponification of $[^{13}C_6]$**7** bis(*tert*-butyl ester), thus suggesting that the peptide bond was also cleaved to some extent. This was confirmed by subjecting the unprotected glutamic acid conjugate DL-**7** (see below) to the same mild alkaline conditions and is in strong contrast to the common belief that boiling in 7 N NaOH for 3 h is necessary to hydrolyze the peptide bonds in *N*-(indol-3-ylacetyl)amino acids of biological importance. A side reaction surprisingly difficult to control when working on a fifty-micromol scale was the formation of *n*-butyl esters (identified by FAB HPLC/MS) when the unprotected aspartic and glutamic acid conjugates were partitioned into *n*-butanol. A search for alternative solvents was insuccessful.

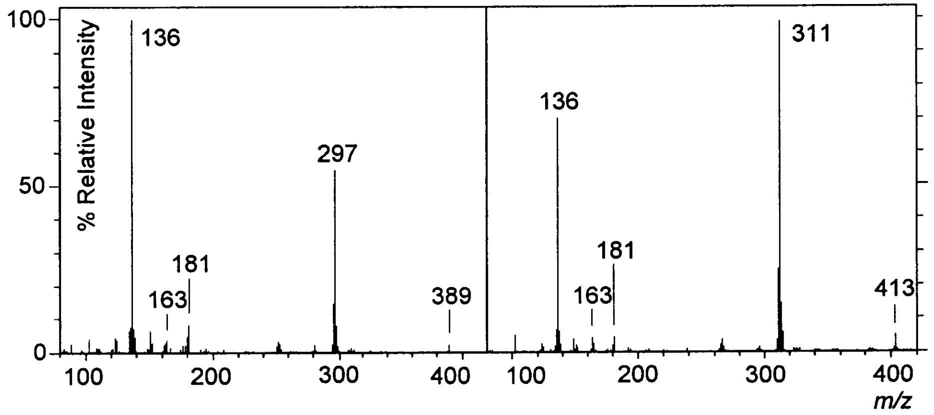
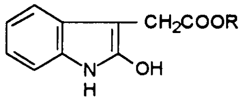


Fig. 2. Mass spectra of *N*-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-aspartic acid (left) and *N*-([3a,4,5,6,7,7a-$^{13}C_6$]indol-3-ylacetyl)-L-glutamic acid (right) obtained by FAB HPLC/MS using a glycerol matrix and ionization by a beam of 5 kV xenon atoms. The fragmentation patterns are discussed in the text.

Conjugates $[^{13}C_6]$**5** and $[^{13}C_6]$**7** were characterized by FAB HPLC/MS using a glycerol matrix (Figure 2). The mass spectra of both labeled amino acid conjugates corresponded closely to those of their unlabeled counterparts (commercial DL-**5**, and DL-**7** prepared as described below) taking into account the appropriate isotope shifts. Specifically, they showed a set of fragments observed for most *N*-(indol-3-ylacetyl)amino acids examined (18, 24): the $[^{13}C_6]$indol-3-ylacetamidium (*m/z* 181), $[^{13}C_6]$quinolinium (*m/z* 136), and $[^{13}C_6]$indolium (*m/z* 123) ions, as well as a fragment (*m/z* 163) generated by elimination of the entire amino acid moiety. Aspartic acid conjugate $[^{13}C_6]$**5** also produced ions at *m/z* 297 $[M + H]^+$, 389 $[M + \text{glycerol} + H]^+$, and 282 $[M - CO_2]^+$, which were shifted by 14 mass units in the spectrum of the glutamic acid derivative $[^{13}C_6]$**7**. Fragments identifying the amino acid moieties were more reliably observed for the dimethyl esters (spectra not shown), *i.e.* *m/z* 162 ($[\text{Asp(OMe)}_2 + H]^+$) and 102 ($[H_2N{=}CHCH_2COOCH_3]^+$) for $[^{13}C_6]$**5**$(OMe)_2$, and the corresponding ions extended by an interspaced CH_2 group for $[^{13}C_6]$**7**$(OMe)_2$.

To permit the condensation of acid **2** with the commercially available, unprotected $[^{15}N]$-L-aspartic and $[^{15}N]$-L-glutamic acids, its *N*-hydroxysuccinimide ester (**4**) was considered as an intermediate. The corresponding ester **3** (19) has already been used for condensations with ω-amino acids (20) and we now also found it suitable for the synthesis of the aspartic and glutamic acid conjugates DL-**5** and DL-**7**. The procedure used in the latter case is outlined in the Experimental section, as the methods so far reported for the preparation of the important plant metabolite **7** (22 - 26) are incompletely documented and, as far as discernible, did not produce consistent results.

1,3-Dicyclohexylcarbodiimide-catalyzed coupling of acid **2** and *N*-hydroxysuccinimide (HOSu) afforded ester **4**, as evidenced by the UV spectrum (absorbance maximum at 250 nm characteristic for 3-substituted 2,3-dihydro-2-oxoindoles) (21) and the following prominent ions in the mass spectrum: *m/z* 289 [M + H]$^+$, 174 [M - OSu]$^+$, 146 [2-hydroxyquinolinium]$^+$, and an ion cluster at 132 - 134 amu of the likely general structure [dihydro-2-oxoindolium + (0 - 2) H]$^+$. Compound **4** then reacted smoothly with both [^{15}N]-L-aspartic and [^{15}N]-L-glutamic acids to yield conjugates [^{15}N]**6** and [^{15}N]**8**. As the residue of **2** comprises roughly equal amounts of two optical isomers (asymmetric carbon at C-3; Figure 1), two diastereomers are formed on conjugation with an L-amino acid. These were, in the case of [^{15}N]**6** and [^{15}N]**8**, separated by HPLC. When isolated, however, the individual diastereomers reequilibrated to the original mixture of isomers, supposedly *via* enol **9** as suggested by the rapid exchange of H-3 for deuterium, in solutions of **6** in CD$_3$OD (14):



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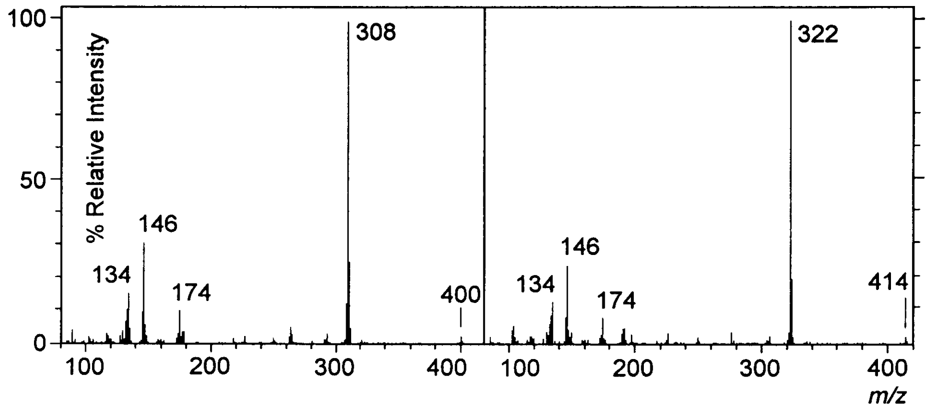

Fig. 3. Mass spectra of [^{15}N]*N*-((*R,S*)-2,3-dihydro-2-oxoindol-3-ylacetyl)-L-aspartic acid (left) and [^{15}N]*N*-((*R,S*)-2,3-dihydro-2-oxoindol-3-ylacetyl)-L-glutamic acid (right) obtained by FAB HPLC/MS (see legend of Figure 2 for conditions). The fragmentation patterns are discussed in the text.

Both diastereomers gave identical mass spectra (Figure 3), *i. e.*, for [^{15}N]**6**: *m/z* 308 [M + H]$^+$, 400 [M + glycerol + H]$^+$, 292 [M - O + H]$^+$, 263 [M - CO$_2$]$^+$, and the corresponding ions shifted by 14 mass units for [^{15}N]**8**. The two conjugates also showed the characteristic fragments derived from the 2,3-dihydro-2-oxo-indol-3-ylacetyl moiety discussed for ester **4**. The dimethyl esters afforded ions derived from the ^{15}N-labeled amino acid moieties, in close analogy to the fragments described for [^{13}C$_6$]**5**(OMe)$_2$ and [^{13}C$_6$]**7**(OMe)$_2$.

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