N- ACYLAMINO ACIDS AND PEPTIDES
III. THE SYNTHESIS OF N- (FERULOYL-2-^{14}C)-GLYCINE-2-^{3}H.

H. De Pooter, I. Pé and C.F. Van Sumere★
★ State University of Ghent, Laboratory of Plant Biochemistry, Ghent, Belgium.
Received on November 8, 1973.

SUMMARY

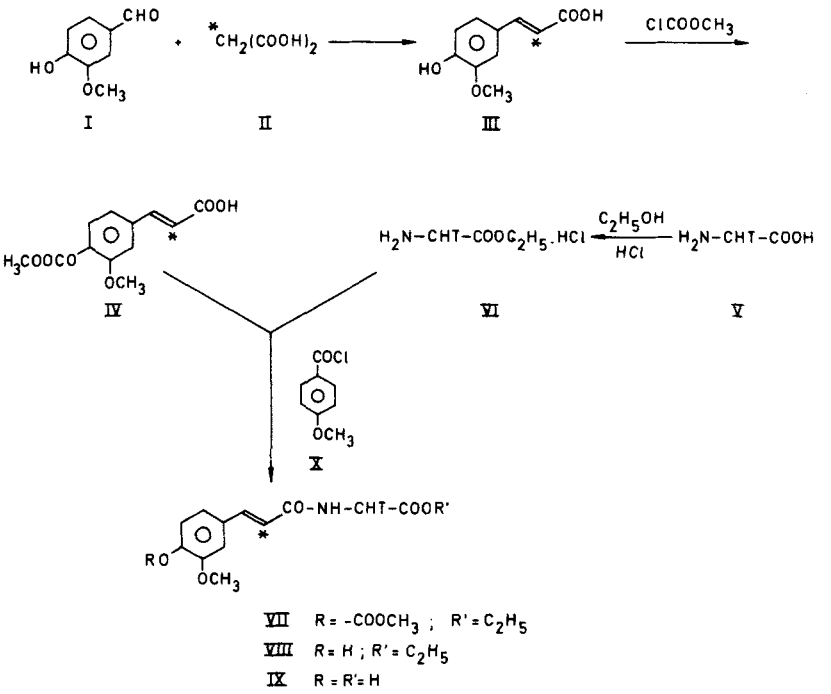
N-(Feruloyl-2-^{14}C)-glycine-2-^{3}H (IX) was obtained with an acceptable yield (43%) starting from O-methoxycarbonyl-ferulic acid-2-^{14}C (IV)and glycine-2-^{3}H ethyl ester (VI). The latter two compounds were first condensed into N-(O-methoxycarbonyl-feruloyl-2-^{14}C)-glycine -2-^{3}H ethyl ester (VII) using anisoyl chloride (X) and the mixed anhydride method (7). Subsequently VII was transformed into N-(feruloyl-2-^{14}C)-glycine-2-^{3}H (IX) by alkaline hydrolysis. The specific activities of the compound were respectively 12.64 µCi (^{3}H)/µmole and 1.71 µCi (^{14}C)/µmole.

The identification of N-feruloyl-glycyl-L-phenylalanine as a sequence in barley proteins (1) raised the question how [and when] the feruloyl-moiety is introduced into the protein, and what role is played by ferulic acid and [or] N-feruloyl-glycine in protein biosynthesis. In view of studying these problems by incorporation experiments with labelled ferulic acid and derivatives, the synthesis of N-(feruloyl-2-^{14}C)-glycine-2-^{3}H (IX) was undertaken.

In theory several procedures are available for the preparation of IX, consisting fundamentally of condensing suitably protected, "activated" ferulic acid with glycine ethyl ester, followed by removal of the blocking groups. The use of O-methoxycarbonyl-feruloyl-2-^{14}C chloride (2,3) was abandoned as, in our hands, the preparation of the compound proved to be erratic. On comparing the condensation by the N,N'-dicyclohexylcarbodiimide- (DCC)(2,4,5), N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline- (EEDQ)(6) or the

mixed anhydride method with ethyl chloroformate or anisoyl chloride (X)(7), the last procedure gave the best yields.

The preparation was then performed as depicted in the scheme.



Ferulic acid-2-^{14}C (III) was obtained by a Doebner condensation of an excess of vanilline (I) with malonic acid-2-^{14}C (II), by adapting the method of Vorsatz (8). After protection of the phenol function with methyl chloroformate, the resulting compound IV was allowed to react with glycine-2-^{3}H ethyl ester (VI)(9,10) via the mixed anhydride method using anisoyl chloride (X)(7). On careful alkaline hydrolysis, the intermediate VII was transformed into VIII, and then into the desired substance IX by repeating the alkaline hydrolysis. The compound, purified by repeated recrystallizations from water, proved to be chemically and radiochemically

pure. The yield of the first (undiluted) batch amounted to 43%, while the total radiochemical yield was 62%. The observed specific activities were 12.64 μCi(^{3}H)/μmole and 1.71 μCi(^{14}C)/μmole.

EXPERIMENTAL

The radioactive starting materials glycine-2-^{3}H (V) and malonic acid-2-^{14}C (II) were purchased from "The Radiochemical Centre - Amersham".

The activity of the compounds and reaction mixtures was determined by means of an "Automatic Tri-Carb Liquid Scintillation Spectrometer model 3380" equipped with an "Automatic Activity Analyzer" (Packard).

TLC-analyses were run on silicagel-cellulose plates, after "steaming", with toluene-ethyl formate-formic acid (5:4:1;v:v:v) as eluent (11). The feruloyl-containing substances were detected by their fluorescence under UV-light of 366 nm, before and after spraying with 2N NaOH.

Autoradiograms were obtained with Agfa-Gevaert Structurix D7 films.

Ferulic acid-2-^{14}C (III)

An aqueous solution of sodium malonate-2-^{14}C (II)(6 mg acid), "diluted" with malonic acid (42 mg), was first weakly acidified with 0.1N HCl (0.6 ml), and then lyophilized. The residual substance (48 mg; 0.46 mmole; spec. activ.: 2.40 μCi/μmole), containing some salt, was further thoroughly dried over P_2O_5 under reduced pressure, and then dissolved in a mixture of 0.4 ml dry pyridine and one drop of dry piperidine. Thereafter, an excess of vanilline (140 mg; 0.92 mmole) was added and the mixture was stirred in darkness for 12 days at ambient temperature. Subsequently the solution was diluted with 0.5 ml H_2O, and acidified to pH = ± 3 with 2N HCl. The yellowish to brownish precipitate formed was separated from the supernatant by suction through a filter stick (porosity 3) and the residual powder was purified by 3 consecutive recrystallizations from H_2O (3 x 2 ml). Dissolved ferulic acid-2-^{14}C was recovered from the mother liquors by addition of "cold" ferulic acid.

Yield (first crop): 41 mg (46%); spec. activ.: 2.26 μCi(^{14}C)/μmole.
Total radiochemical yield : 75%

TLC-analysis: one blue fluorescent spot with Rf = 0.59, corresponding to ferulic acid (11); negative reaction when sprayed with 2,4-dinitrophenylhydrazine. The autoradiogram showed one spot, Rf = 0.59.

O-Methoxycarbonyl-ferulic acid-2-^{14}C (IV)

A mixture of ferulic acid-2-^{14}C (III)(15.1 mg; spec. activ.: 2.26 µCi/µmole) and ferulic acid (4.3 mg)(total III: 19.4 mg; 0.1 mmole; spec. activ.: 1.76 µCi/µmole) was dissolved at 0°C in 2.03 ml 0.1N NaOH in a two neck conical reaction flask of 50 ml. After addition of 10 µl methyl chloroformate (0.12 mmole), the yellowish solution was vigorously stirred with a mechanical micro-stirrer for 1 h. A second batch of methyl chloroformate (3 µl) was then added, and stirring was continued for an additional hour. Thereafter the reaction mixture was acidified with 2N HCl (0.2 ml) and the voluminous precipitate formed was isolated, after cooling at -5°C, by means of a filter stick (porosity 3). The resulting powder was then washed with 3 ml H_2O, thoroughly dried over P_2O_5 in vacuo and further used as such. (TLC-analysis of the compound, isolated from a test-run, indicated complete absence of ferulic acid. However the presence of a small amount of a contaminating substance, which was tentatively identified as the mixed anhydride of IV and methyl hydrogen carbonate, was detected. Since this compound did not interfere with the subsequent reaction, no steps were taken to remove it.)

Glycine-2-^{3}H ethyl ester hydrochloride (VI)

A 2.5 ml aqueous solution of glycine-2-^{3}H was diluted with "cold" glycine (8 mg; 0.107 mmole) in a conical 25 ml flask (final spec. activ.: 15.98 µCi(^{3}H)/µmole). Thereafter the solution was concentrated to dryness by lyophilisation, and the resulting, cotton-like glycine-2-^{3}H was thoroughly dried over P_2O_5 in vacuo. The compound was then dissolved in a mixture of 1.8 ml dry ethanol and 0.2 ml ethanol saturated with HCl-gas. Subsequently the solution was gently boiled for 30 min. and 7.8 µl acetyl chloride was added (9). Boiling was further continued for 1 h. Thereafter the solvent was distilled off under reduced pressure. The residual crystals were treated with ether (3 ml) and the ether removed by distillation. This treatment was repeated three times. Finally the

isolated compound was dried over P_2O_5. In order to obtain complete transformation into VI, the esterification was once repeated (cfr (10)).

N-(Feruloyl-2-^{14}C)-glycine-2-^{3}H (IX)

O-Methoxycarbonyl-ferulic acid-2-^{14}C (IV), prepared as described above, was dissolved in dry methylene chloride (3 ml), containing one drop of dry triethylamine. After cooling to -10°C a solution of anisoylchloride (17 mg; 0.1 mmole) in dioxane (± 0.5 ml) and methylene chloride (1 ml) was added by means of a separating funnel. The reaction mixture was subsequently stirred at -10°C for 1 h, and then cooled to -20°C. Meanwhile, glycine-2-^{3}H ethyl ester hydrochloride (VI - see above), dissolved in 2 ml methylene chloride containing 100 µl triethylamine, was transferred in dry circumstances into the separating funnel (mounted on the feruloyl-containing reaction flask), and added to the mixed anhydride. The flask which contained the labelled glycine ethyl ester was 3 times washed with 2 ml methylene chloride and the pooled washings were added to the reaction mixture. After a period of 2 h at -20°C, the solution was kept overnight at 0°C, and then concentrated to 0.5 ml under reduced pressure. The residue obtained was distributed between ethyl acetate (10 ml) and 0.1N HCl -5% NH_4Cl (5 ml). The water layer was removed by suction, and the residual organic layer was successively washed with 5% KCl (5 ml), 0.1M K_2CO_3 - 5% KCl (5 ml) and 5% KCl (5 ml). After concentration to dryness in vacuo, the residue was further dissolved in 2 ml ether. The solvent was removed by distillation (procedure 3 times repeated) and the residue stirred for 15 min. at ambient temperature in 2 ml methanol containing 0.2 ml 1N KOH. Thereafter, the reaction mixture was neutralized with 0.2 ml 1N HCl and concentrated to 0.5 ml in vacuo. The aqueous residue was diluted with 1 ml 4N KOH at 0°C and quickly extracted with ethyl acetate (2 x 3 ml) and ether (3 ml). Subsequently, the alkaline layer was heated for 10 min. at 70° - 75°C. After cooling to room temperature, the yellow solution was carefully decolourized with 4N HCl, and then brought to pH = 5-6 with 1M $NaHCO_3$ (± 0.2 ml). The water layer was then successively extracted with ethyl acetate (2 x 5 ml) and ether (5 ml), strongly acidified with conc. HCl (0.5 ml), saturated with NH_4Cl, and finally thoroughly extracted with ethyl acetate (5 x

5 ml). The organic extract was concentrated to dryness and the residue was purified by 3 consecutive recrystallizations from H_2O (each 0.5 - 1 ml). Dissolved radioactivity was recovered by recrystallizing "cold" N-feruloyl-glycine from the mother liquors. (Note: if the originally isolated, crude IX is in the form of a half-crystalline mass, the recrystallizations are preceded by a treatment with ethyl acetate - petroleum ether (1:1; v:v; 3 ml), which on removal leaves a lightly yellowish powder.)

Yield (first crop): 10.79 mg (43%); spec. activ.: 12.64 μCi(^{3}H)/μmole and 1.71 μCi(^{14}C)/μmole.

Total radiochemical yield: 62% (calculated on ^{14}C).

TLC-analysis: when observed under UV-light of 366 nm, the TL-plate showed one blue-fluorescing spot (Rf = 0.41) which turned greenish-blue after spraying with 2N NaOH. Further treatment with diazotized p.nitroaniline (see (11) and references therein) gave rise to a slate-blue colour. The foregoing TLC-characteristics are typical for N-feruloyl-glycine (2).
The radiochemical purity of the labelled substance was furthermore proven by autoradiography.

Acknowledgement

The support of our work by the Belgian "Fonds voor Kollektief Fundamenteel Onderzoek" is gratefully acknowledged.

References

(1) Van Sumere C.F., De Pooter H., Ali M. and Degrauw-Van Bussel M. - Phytochem. 12 : 407 (1973).

(2) De Pooter H., Ali M. and Van Sumere C.F. - Bull. Soc. Chim. Belges 82 : 243 (1973).

(3) Fischer E. and Hoesch K. - Ann. Chem. 391 : 347 (1912).

(4) Sheehan J.C. and Hess G.P. - J. Am. Chem. Soc. 77 : 1067 (1955).

(5) Booth A.N., Emerson O.H., Jones F.T. and DeEds F. - J. Biol. Chem. 229 : 51 (1957).

(6) Belleau B. and Malek G. - J. Am. Chem. Soc. 90 : 1651 (1968).

(7) Birr C., Lochinger W. and Wieland T. - Ann. Chem. 729 : 213 (1969).
(8) Versatz F. - J. prakt. Chemie 145 : 265 (1936).
(9) Baker B.R., Schaub R.E., Querry M.V. and Williams J.H. - J. Org. Chem. 17 : 97 (1952).
(10) Herbert M., Jimeno Osso F. and Pichat L. - J. Labelled Comp. VI. · 45 (1972).
(11) Van Sumere C.F., Wolf , Teuchy H. and Kint J. - J. Chromatogr. : 48 (1965).