SYNTHESIS OF ^{14}C-OCHRATOXIN A

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SUMMARY

^{14}C-Ochratoxin A is obtained by coupling the N-hydroxysuccinimide derivative of the lactone acid hydrolysate of natural ochratoxin A with ^{14}C-L-β-phenylalanine methylester. ^{14}C-Ochratoxin A methylester is converted into ^{14}C-ochratoxin A by alkaline hydrolysis. Purification of the intermediates and the ^{14}C-ochratoxin A is achieved by HPLC. For an amount of 0.05 µmole L-β-phenylalanine methylester the yield of ochratoxin A is 0.02 µmole or 40 % and the specific activity 130 Ci/mole. Stereospecificity is 100 % and radiochemical purity is 96 %.

The yield of ^{14}C-ochratoxin A increases with increasing amounts of ^{14}C-L-β-phenylalanine methylester and reaches 80% under the best conditions.

Key Words : ^{14}C-ochratoxin A-synthesis-amide bond

INTRODUCTION

The ochratoxins constitute a group of chemically closely related secondary metabolites produced by species of the genera Aspergillus and Penicillium. They are isocoumarin compounds linked by an amide bond to L-β-phenylalanine. The ochratoxins and the corresponding methyl and ethylesters have been isolated from fungal cultures. Beside nephropathies they cause liver disease and oncogenic and teratogenic effects in test animals (1,2). Ochratoxin A (Fig. 1) is the major metabolite with respect to both occurrence and toxicity. The presence of this

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0362-4803/84/050429–12$01.20

Received November 10, 1983

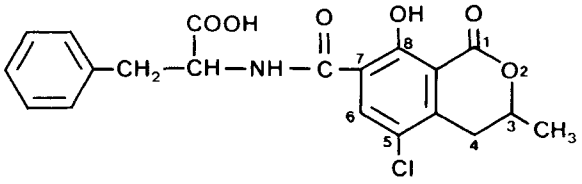

Fig. 1. Ochratoxin A

toxin in food products for human and animal consumption has led to an increased interest in the development of rapid and sensitive techniques (3,4).

Radioimmunoassay (RIA) is such a rapid, simple and sensitive method. Thus far to our knowledge only one RIA, using ^{3}H-ochratoxin A, has been described (5). The preparation of ^{3}H-ochratoxin A was achieved by hydrogen exchange reaction (6). The radiochemical yield was rather low and long-term stability of the labeled compound was not thoroughly investigated.

The purpose of the present study was to synthesize a ^{14}C-ochratoxin A with a high specific activity and a high stability.

Because L-[U-^{14}C]-β-phenylalanine with sufficient specific activity is commercially available we have investigated the coupling of the N-hydroxysuccinimide (NHS) derivative of the lactone acid hydrolysate of ochratoxin A with ^{14}C-L-β-phenylalanine methylester via an amide bond.

The lactone acid is obtained by acid hydrolysis of natural ochratoxin A. It is isolated by extraction in chloroform. Subsequently the lactone acid is coupled with N-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC). The NHS-lactone derivative is treated with ^{14}C-L-β-phenylalanine methylester in dry pyridine to form ^{14}C-ochratoxin A methylester, which is then converted into ^{14}C-ochratoxin A by alkaline hydrolysis.

MATERIALS

Ochratoxin A is obtained from Janssen Chimica. L-[U-^{14}C]-phenylalanine is purchased from Amersham International. Specific activity is 496 Ci/mole. All other chemicals are either analytical grade or chemically pure.

Cellulose thin-layer plates (thickness 0.1 mm) and Silica gel 60 thin-layer plates (thickness 0.25 mm) are obtained from Merck.

A model 1040 Pye Unicam high pressure liquid chromatograph with a RP-18 Lichroma column, 250 by 4 mm, particle size 10μ, with a model 1020 Pye Unicam U.V. and a Pye Unicam fluorescence detector (excitation filters 7-54 + 7-60 -

emission filters 4-76 + 3-72) and a Pharmacia Fine Chemicals Fraction Collector FRAC-100 is used for analytical and preparative separations.

The radioactivity measurements are carried out with a Packard Tri-Carb Model 3390 liquid scintillation spectrometer.

EXPERIMENTAL

Preparation of lactone acid

The lactone acid is obtained by acid hydrolysis of natural ochratoxin A. Ochratoxin A (15 mg) is suspended in 25 ml concentrated HCl and refluxed for 28 hrs under nitrogen. Chloroform extraction of the mixture yields the lactone acid or 5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin-7-carboxylic acid as described by Van Der Merwe et al. (7).

Prior to chloroform extraction a known amount of L-[U-^{14}C]-phenylalanine is added to the hydrolysate to check the quantitative elimination of L-β-phenylalanine. Washing of the chloroform phase with water is continued until no further ^{14}C-activity in the organic phase is detected.

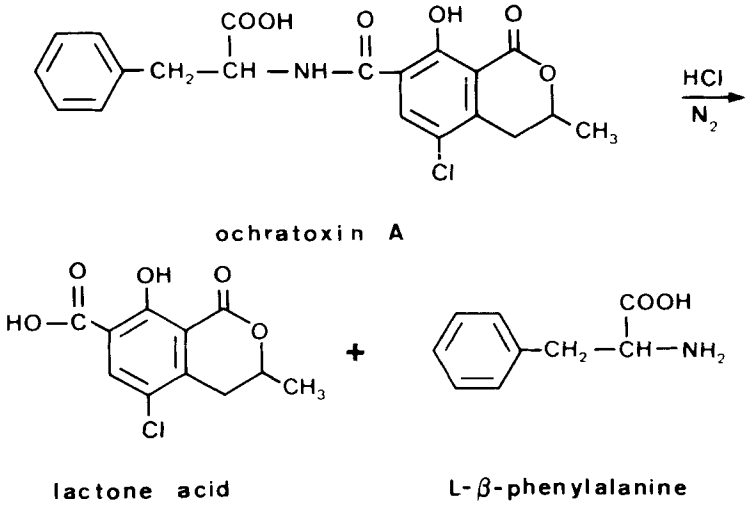

Fig. 2. Hydrolysis of ochratoxin A

The elimination of L-β-phenylalanine is also checked in the aqueous phase by TLC. The aqueous phase is adjusted to pH 5.8 with NaOH. A known fraction is spotted on a cellulose plate. The plate is developed in butanol : water : acetic acid (4:1:1, v/v), dried, sprayed with ninhydrin reagent and heated at 110 °C for 10 min. Spots are compared with standards treated in the same way as the water phase.

After elimination of L-β-phenylalanine the chloroform is evaporated with a nitrogen stream at room-temperature. The yield is 9.5 mg.

Preparation of N-hydroxysuccinimide derivative of the lactone acid

9.5 mg lactone acid is treated with 142.5 mg NHS and 285 mg DCC in 1 ml dry dioxane at 4 °C for 1/2 hr and subsequently at room-temperature for 12 hrs. The reaction is shown in Fig. 3.

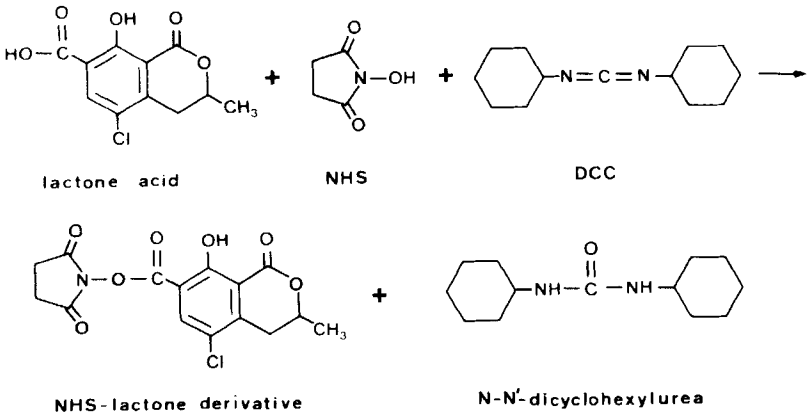

Fig. 3. Preparation of NHS-lactone derivative

The N-N'-dicyclohexylurea precipitates. Centrifugation is carried out and the supernatant, which contains the NHS-lactone derivative is separated. To collect all traces of NHS-lactone derivative the precipitate is washed twice with 0.5 ml dry dioxane and centrifuged. All dioxane portions are pooled (total volume 2 ml) and injected in several 100 µl fractions on a RP-18 HPLC column in order to separate the NHS-lactone derivative from the excess of NHS and DCC. The eluates are collected with a fraction collector. The solvent is methanol :

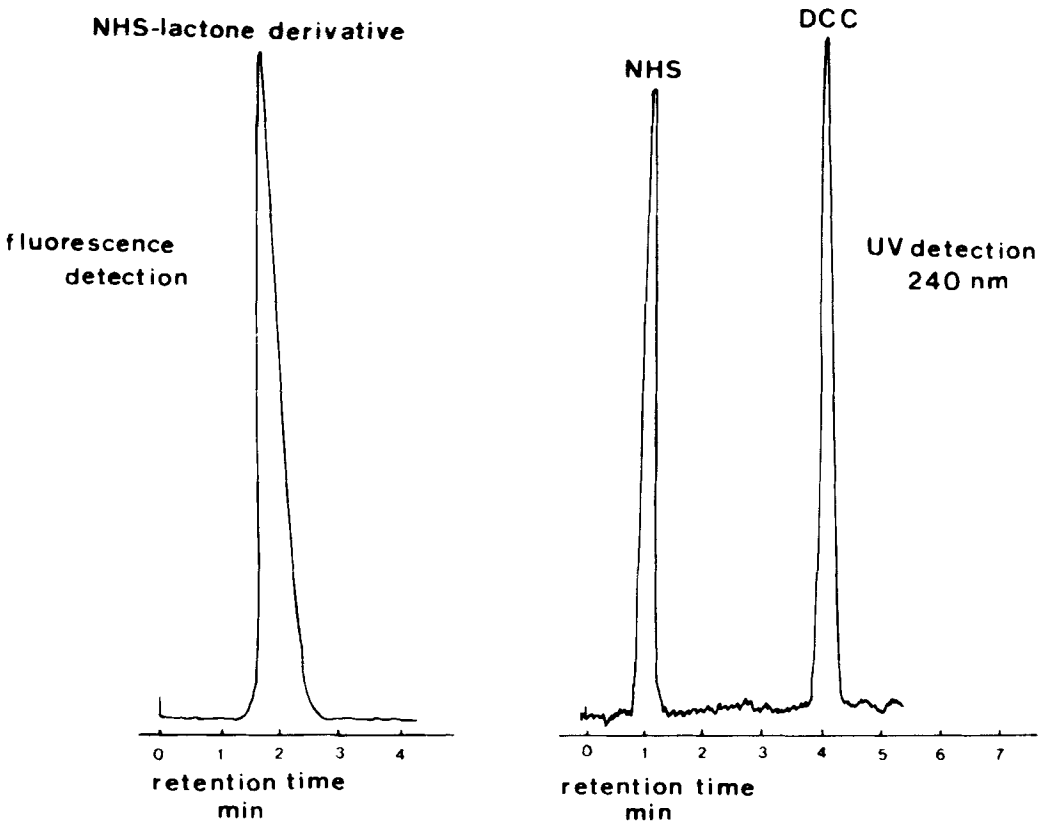

Fig. 4. Chromatogram of NHS-lactone derivative, NHS and DCC

water : acetic acid (70:30:1, v/v) at a flow rate of 1.3 ml/min. The NHS-lactone derivative is collected from 1.8 to 3.4 ml (± 2 min). The solvent is evaporated in a nitrogen stream at room-temperature. NHS and DCC can not be detected with a fluorescence detector. U.V. detection gives a NHS-peak at about 1 min. and a DCC-peak at about 4 min (Fig. 4).

Preparation of ^{14}C-L-β-phenylalanine methylester

L-[U-^{14}C] phenylalanine (0.05 μmole) is treated with 8 ml of a mixture of methanol and thionylchloride (10:1, v/v) at 0 °C for 1 hr and subsequently kept for another 16 hrs at room-temperature after which the reaction is completed. The solution is evaporated in vacuum at room-temperature.

The esterification process is verified by spotting an aliquot of the reaction mixture on a cellulose TLC plate and developing the plate in methanol:water:pyridine (80:20:4, v/v). A good separation between L-ß-phenylalanine and the corresponding methylester is obtained (spots are visible under U.V. light). To determine the end of the esterification process accurately the spots are scraped off and counted for radioactivity.

Preparation of ^{14}C-ochratoxin A

The NHS-lactone derivative is dissolved in 100 µl dry pyridine and cooled at 4 °C. This solution is added to 0.05 µmole ^{14}C-L-ß-phenylalanine methylester and the mixture is shaken for 24 hrs at 4 °C.

To convert ^{14}C-ochratoxin A methylester into ^{14}C-ochratoxin A 40 µl 1 N NaOH is added to the solution (Fig. 5). After 1 hr at 4 °C the reaction is completed and the solution injected in the HPLC equipped with a RP-18 column and a fraction collector. The ^{14}C-ochratoxin A peak is collected and the solvent evaporated in vacuum at room-temperature. The final product is dissolved in 10 ml benzene.

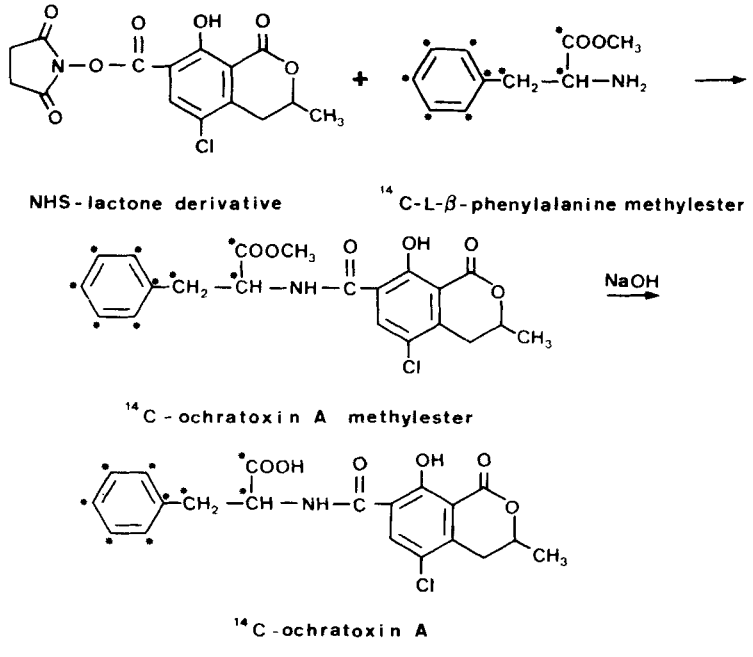

Fig. 5. ^{14}C-ochratoxin A synthesis

*Possible location of label

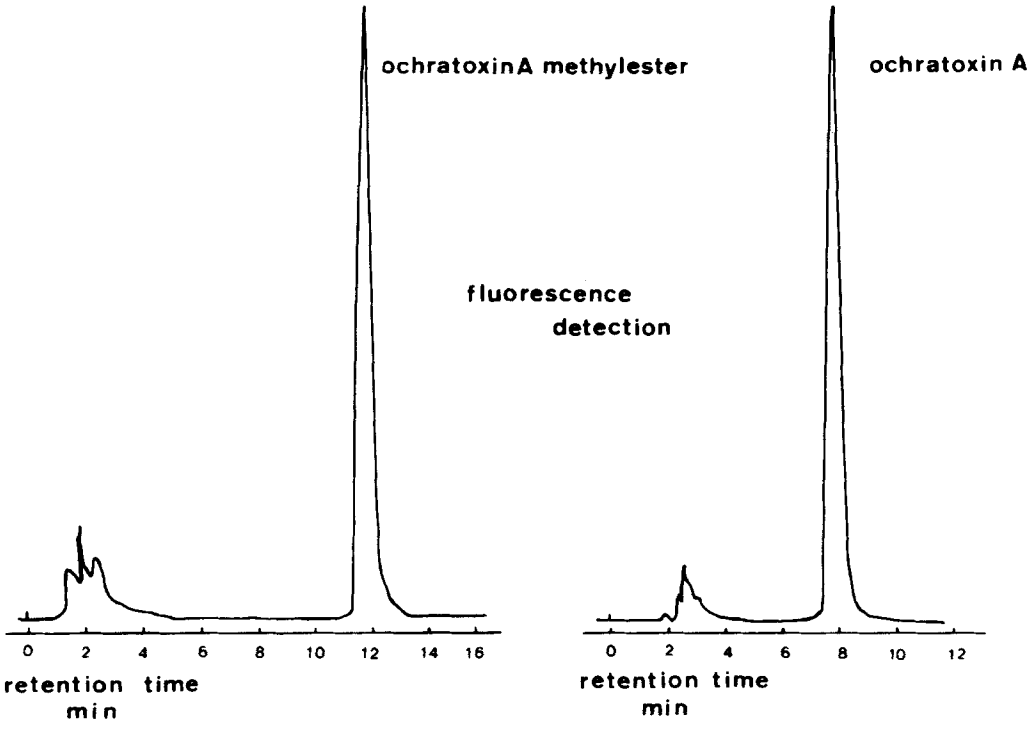

Fig. 6. Chromatogram of ^{14}C-ochratoxin A methylester and ^{14}C-ochratoxin A. Solvent : methanol : water : acetic acid (70:30:1, v/v) flow rate 1.3 ml/min.

The alkaline hydrolysis is followed by injecting fractions of the reaction mixture in the HPLC. ^{14}C-ochratoxin A and the corresponding methylester are completely separated (Fig. 6).

RESULTS

Stereospecificity of ^{14}C-ochratoxin A

When the synthesis of ochratoxin A is carried out with a racemic mixture of DL-β-phenylalanine instead of L-β-phenylalanine two peaks are obtained for ochratoxin A (Fig. 7).

Fig. 6 shows only one peak of ^{14}C-ochratoxin A. So it can be concluded that the ochratoxin A is 100 % stereospecific .

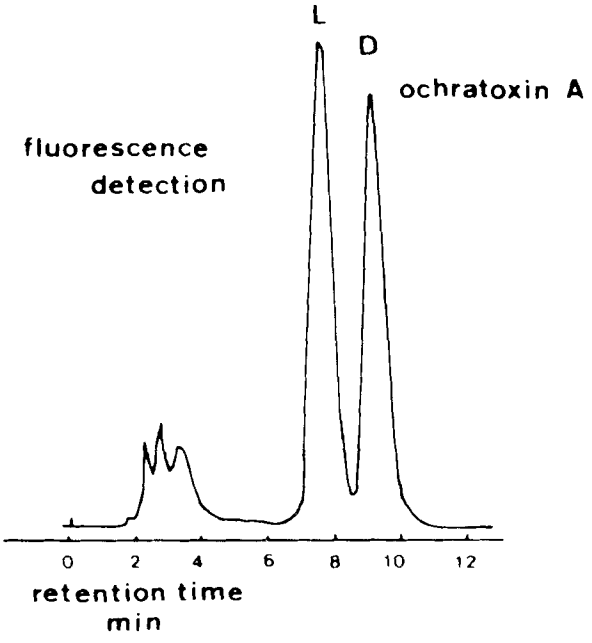

Fig. 7. Chromatogram of DL ochratoxin A. Solvent : methanol : water : acetic acid (70:30:1, v/v) flow rate 1.3 ml/min.

Radiochemical purity

Radiochemical purity is determined by TLC on silica gel with a mixture of benzene : methanol : acetic acid (80:10:10, v/v) as solvent. From the data of the chromatogram the radiochemical purity is calculated as 96 %.

Specific activity

The carrier content of the final solution is determined by HPLC on a RP-18 column and with fluorescence detection. It is calculated from a calibration curve and found to be 0.02 μmole .

Absolute activity of the final ochratoxin A solution is determined by liquid scintillation counting. A calibrated sample of ^{14}C-toluene is used as the internal standard. An absolute activity of 2.6 μCi is found. So the specific activity is 130 Ci/mole.

Total yield

The total yield of 40 % is calculated from the amount of ^{14}C-L-β-phenylalanine (0.05 μmole) and the amount of ^{14}C-ochratoxin A.

Stability

The synthesized ^{14}C-ochratoxin A (0.02 μmole) is dissolved in 10 ml benzene and stored at 0 °C. In these conditions the solution is stable for several months. This is checked by HPLC with the same parameters as described in Fig. 6.

DISCUSSION

Preparation of N-hydroxysuccinimide derivative of the lactone acid

The formation of the N-hydroxysuccinimide derivative of the lactone acid was tested not only in dry dioxane but in methanol, ethylacetate, pyridine and dimethylformamide as well. The highest yield of NHS-lactone derivative was obtained in dioxane.

It was also shown that proceeding the reaction at room-temperature gives rise to increased hydrolysis of the NHS-lactone derivative.

When the reaction was entirely performed at 4 °C it was not complete.

The influence of an excess of NHS and DCC on the yield of the NHS-lactone derivative was also investigated. The results are shown in Fig. 8. The amount of lactone acid used in the reaction was kept constant at 9.5 mg. The ratio of the amount of NHS to DCC was kept constant at 1/2.

From Fig. 8 it can be concluded that for a given amount of 9.5 mg lactone acid at least 142.5 mg NHS (15-fold excess) and 285 mg DCC (30-fold excess) are needed to obtain a maximal yield. HPLC purification of the NHS-lactone derivative is necessary to remove the excess of NHS and DCC in order to avoid cross-reactions in the next synthesis steps.

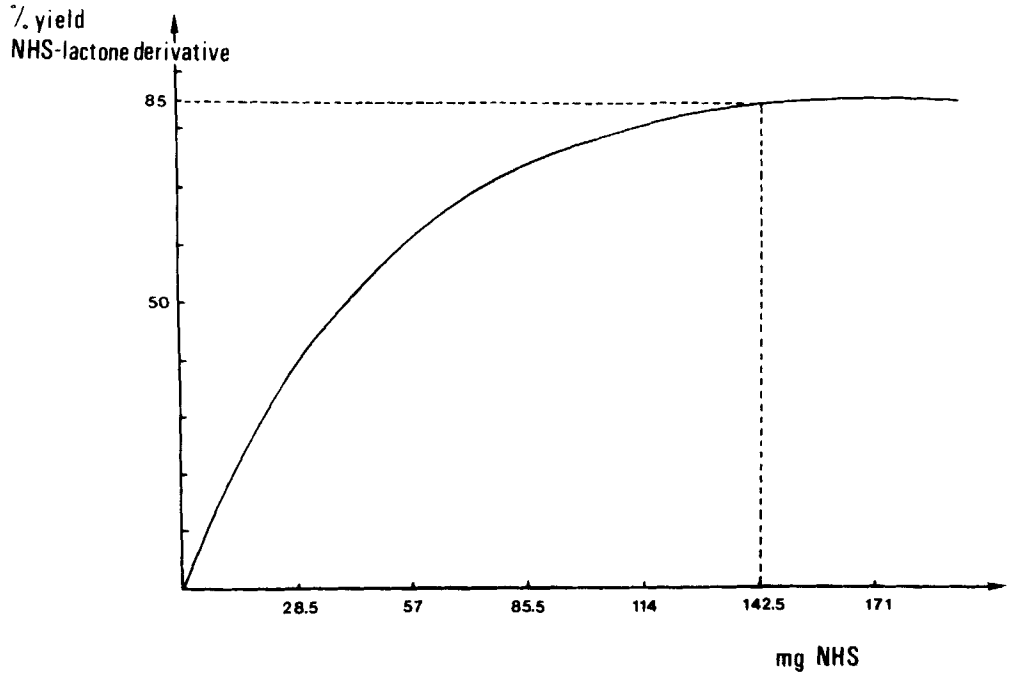

Fig. 8. Influence of the excess of NHS and DCC on the yield of the NHS-lactone derivative. The amount of NHS to DCC is 1/2.

Preparation of ^{14}C-ochratoxin A

When ^{14}C-L-β-phenylalanine was used instead of the corresponding methylester in the reaction with the NHS-lactone derivative, the yield of ^{14}C-ochratoxin A decreased significantly. This phenomenon is very probably due to the free carboxyl group in L-β-phenylalanine which may interfere in the coupling reaction. Also the solubility of L-β-phenylalanine in pyridine is inferior to that of the methylester.

The coupling reaction of the NHS-lactone derivative with L-β-phenylalanine methylester was compared in methanol, ethylacetate, pyridine, chloroform, dioxane and dimethylformamide. The best results were obtained in pyridine.

The influence of an excess of NHS-lactone derivative and the influence of the amount of L-β-phenylalanine on the yield of ochratoxin A was investigated. The results are shown in Fig. 9.

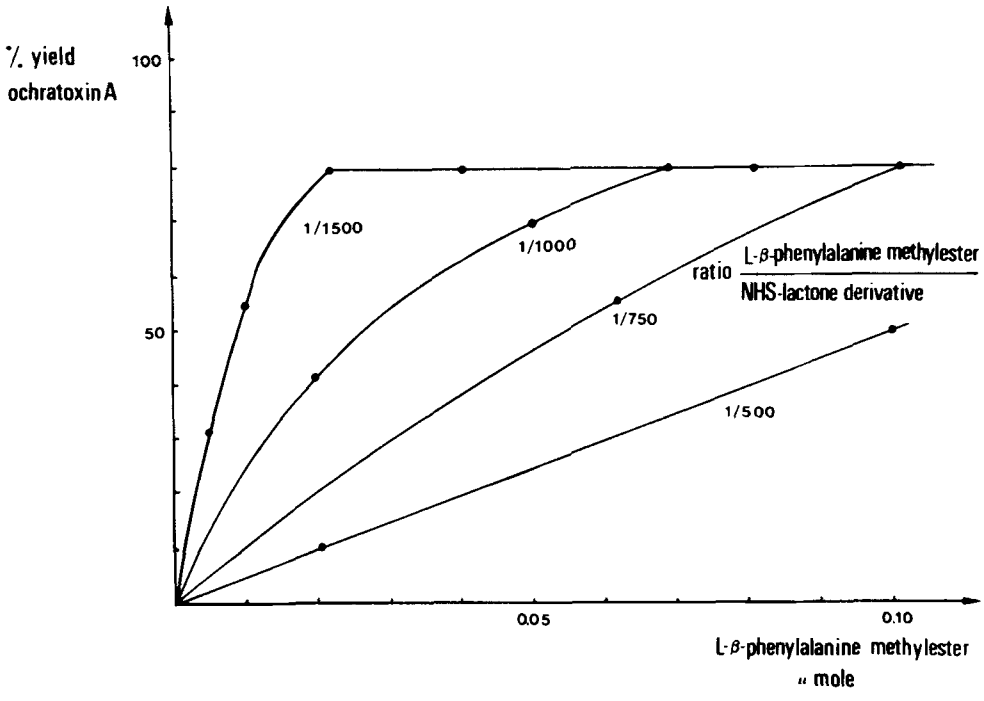

Fig. 9. % yield of ochratoxin A versus amount L-β-phenylalanine methylester with varying ratios L-β-phenylalaninemethylester to NHS-lactone derivative.

From Fig. 9 it can be concluded that for a given amount of ^{14}C-L-β-phenylalanine methylester of 0.05 µmole at least a 1500 fold excess of NHS-lactone derivative is necessary to obtain 80 % ochratoxin A.

We have chosen an amount of 0.05 µmole L-[U-^{14}C] phenylalanine and 9.5 mg lactone acid as start material to synthesize 2.6 µCi ^{14}C-ochratoxin A, which was in our work a sufficient amount of radioactivity to start the development of a radioimmunoassay.

With increasing amounts of ^{14}C-L-β-phenylalanine methylester and increasing amounts of NHS-lactone derivative the yield of ^{14}C-ochratoxin A can reach 80 %.

ACKNOWLEDGEMENTS

This work was supported by the NFWO (grant 3.0033.83) and the I.W.O.N.L.

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