The Synthesis of Specifically ^{14}C-Labelled 2,6-Diaminopimelic Acid and its Higher Homologues

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SUMMARY

D,L-2,6-Diaminopimelic-(2-^{14}C) acid was prepared by alkylation of diethyl acetamidomalonate-(2-^{14}C) with 5-bromo-N-phthaloyl-L-norvaline methyl ester and hydrolysis of the condensation product. Depending on the alkylation conditions, partial or complete racemisation takes place and therefore the L,L-form of diaminopimelic acid had to be isolated by paper chromatography. Alkylation of diethyl acetamidomalonate-(2-^{14}C) with diethyl 4-bromobutyl- or 5-bromopentyl- or 6-bromohexylacetamidomalonate and subsequent hydrolysis of the condensation products gave D,L-2,7-diaminosuberic-(2-^{14}C), D,L-2,8-diaminoazelaic-2-^{14}C, and D,L-2,8-diaminosebacic-(2-^{14}C) acids respectively.

2,6-Diaminopimelic acid is an important component of the cell wall peptides of micro-organisms. The biosynthesis of these peptides can be conveniently studied by means of labelled precursors and therefore this study is devoted to the preparation of ^{14}C labelled 2,6-diaminopimelic acid and some of its higher homologues labelled specifically with the same isotope.

The first synthesis of non-radioactive D,L-2,6-diaminopimelic acid was published by Sörensen and Andersen (1) before it was isolated by Work (2) from natural material. Their synthesis consists in the reaction of crude diethyl 5-bromopropylphthalimidomalonate with sodium diethylphthalimidomalonate. The reaction product after hydrolysis is, of course, a racemic mixture. Using similar procedure, but employing diethyl acetamidomalonate-(2-^{14}C), Chaloupka and Vereš (3) prepared D,L-2,6-diaminopimelic-(2-^{14}C) acid. Racemic 2,6-diaminopimelic acid labelled in the position 1 was also prepared by Tannenbaum and Kanako (4).

In our synthetic study we tried to condense diethyl 2-acetamido-2-(3′-bromopropyl)malonate prepared by Rothstein and Clause (5) with diethyl acetamidomalonate-(2-^{14}C) with radioactive 2,6-diaminopimelic acid.

However, the yield was low whatever the reaction conditions, because an intramolecular cyclisation to a proline derivative usually prevailed. Moreover, racemic diaminopimelic acid was also a reaction product after hydrolysis. As any natural material contains only LL- and meso-forms of diaminopimelic acid, it was advantageous to prepare this substance for subsequent biological investigations in an optically pure and specifically labelled form. We utilised the methyl ester of 5-bromo-N-phthaloyl-L-norvaline prepared by us (6) because it does not contain a free hydrogen atom in the amino group, is able to cyclise, and because it was suitable for a radioactive synthesis.

We investigated a number of reaction conditions and also, in addition to the sodium salt, the magnesium and lithium salts of diethyl acetamidomalonate. The reactions were carried out on an extremely low scale of radioactivity. The hydrolysed reaction mixtures were chromatographed in methanol-puridine-water (77 : 10 : 20) proposed by Work (7) and Greenstein (8) in which the *meso-* + D-forms separate from the L-form of diaminopimelic acid. Scanning of the chromatograms was used for the evaluation of the degree of racemisation and the yields.

We found that, (*a*) in all instances almost complete or complete racemisation of the resulting diaminopimelic acid took place, and (*b*) the best yields were attained when the sodium salt of diethyl acetamidomalonate-(2-^{14}C) in dimethylformamide was used.

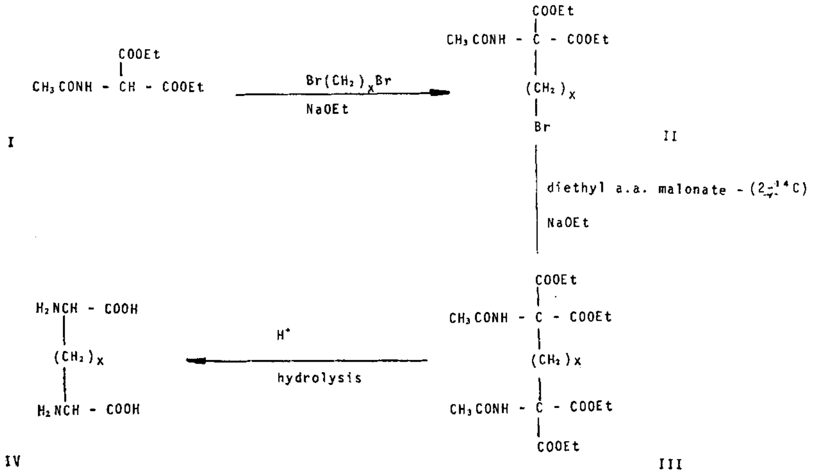
The method (*b*) was employed for the active synthesis, and the racemic mixture of diaminopimelic-(2-^{14}C) acid after hydrolysis was purified and separated on paper Whatman No. 17. This "thick" paper had to be used because on a commonly used preparative paper, Whatman No. 3, a good separation could be observed only when a few milligrams were applied on the whole sheet. A higher loading led to a superposition of zones.

However, the use of Whatman No. 17 paper leads to appreciable losses due to adsorption on the large amount of cellulose.

The preparation of non-radioactive 2,7-diaminosuberic acid and 2,8-diaminoazelaic acid is described in the literature by Simmonds (9). He obtained amino acids IV on condensation of the sodium salt of diethyl acetamidomalonate with 1,4-dibromobutane or 1,5-dibromopentane and subsequent hydrolysis of the reaction product. The reaction intermediates II and III were not isolated. Inactive 2,9-diaminosebacic acid was prepared by amination of 2,9-dibromosebacic acid.

To carry out the radioactive syntheses we made use of a single procedure for all three amino acids IV. Inactive ω-bromoderivatives (II) were prepared by condensation of the sodium salt of diethyl acetamidomalonate (I) with corresponding α,ω-dibromoalkanes and purification of bromides II*a*, *b*, *c* on alumina columns. These bromides II gave on reaction with the sodium salt of diethyl acetamidomalonate-(2-^{14}C) in ethanol "tetraesters" III*a*, *b*, *c* in good yield, but they were not isolated. By direct hydrolysis of the reaction mixture the corresponding C-labelled α,α'-diaminodicarboxylic acids (IV) were obtained. The radioactive synthesis of these amino acids was carried out using lower specific activities which sufficed for the preliminary biological experiments. In order to identify the intermediates, pure "tetraesters" III*a*, *b*, *c* were prepared by another route, i.e. the direct condensation of the sodium salt of diethyl acetamidomalonate with α,ω-dibromoalkanes.

SCHEME



IIa, IIIa, IVa ...... x = 4
IIb, IIIb, IVb ...... x = 5
IIc, IIIc, IVc ...... x = 6

EXPERIMENTAL.

Chromatography, activity measurement.

Chromatography of acids IV*a*, *b*, *c* was carried out in *n*-butanol saturated with 10 % HCl. It was very suitable for checking the radiochemical purity by autoradiography. For the identification of reaction products hydrolysis was followed by chromatography in *n*-butanol-acetic acid-water (4 : 1 : 5) and methanol-pyridine-water (77 : 10 : 20). Autoradiography was carried out in the usual manner, using a fim ORWO, Normal RF_3.

Radioactivity was measured with a scintillation scanner Mark I of Nuclear, Chicago.

2,6-Diaminopimelic Acid-(2-^{14}C).

Diethyl acetamidomalonate-(2-^{14}C) (214.5 mg; 0.989 mmole; spec. act. 6.16 mCi/mmole) of total activity of 6090 μCi was introduced into a 10 ml ground joint test tube and 0.6 ml of abs. ethanol was added to it. The latter was distilled off to dryness under exclusion of humidity and to the residue was added 0.989 mmole of sodium ethoxide in ethanol (0.75 ml). The reaction mixture was concentrated to dryness at normal pressure and the sodium salt of diethyl acetamidomalonate was dried for another 2 hours at 120° C and 15 Torr. The residual solid foam was mixed with 2 mmoles of 5-bromo-2-phthaloyl-L-norvaline methyl ester in abs. ether. The solvent was evaporated and 0.8 ml of abs. dimethylformamide were added to the residue. The mixture was heated slowly under an inert gas to 100-110° C and kept at this temperature until neutral (approx. 4 hours). After cooling, 4 ml of water were added to the mixture which was then extracted continually with ethyl acetate in a small extractor for two hours. The ethyl acetate extract was evaporated to dryness and mixed with 1 ml of conc. HCl and 3 ml of glacial acetic acid and refluxed for 12 hours. The volatile components were evaporated twice upon addition of a few milliliters of water (phthalic acid separated). Finally the aqueous solution was filtered from the phthalic acid and the filtrate was applied on 4 sheets of Whatman No. 17 paper. Repeated ascending chromatography was carried out with methanol-pyridine-water (77 : 10 : 20) (three times). After autoradiographic detection zones corresponding to (*meso* + D,D) and L,L-diaminopimelic acid and glycine were eluted with 3 % ammonia and the eluates were evaporated to dryness. These eluates are free of all ninhydrin-positive impurities and they are also radiochemically pure. However, they contain impurities eluted from the paper. The eluates of (*meso* + D,D) and L,L-diaminopimelic acid were applied on Whatman No. 3 paper and chromatographed in n-butanol-acetic acid-water (4 : 1 : 5) for 20 hours. The zones of both forms were again eluted with 3 % ammonia, decolorized with charcoal, filtered and freeze-dried. L,L-Diaminopimelic acid (380 μCi) (optically pure) and (*meso* + D,D)-diaminopimelic acid (780 μCi) were thus obtained.

The activity balance of the course of the whole experiment is represented in the following table :

Introduced activity (according to certificate)	6,090 μCi
ethyl acetate extract after condensation	5,750 μCi
amount applied on paper Whatman No. 17	5,400 μCi
L,L-diaminopimelic acid eluted	450 μCi
(*meso* + D,D)-diaminopimelic acid eluted	850 μCi
glycine eluted	860 μCi

The yield of the racemic diaminopimelic acid was evaluated by scanning the chromatogram of the hydrolysed condensation product run in methanol-pyridine-water (77 : 10 : 20). This yield was approx. 50 % of the theoretical.

Diethyl 2-Acetamido-2-(4'-bromobutyl)-malonate (IIa).

Diethyl acetamidomalonate (9.8 g; 45 mmole) was added to a solution of 1.04 g (45 mmole) of sodium in 35 ml of abs. ethanol and the mixture was boiled for a short time. Dried, distilled 1,4-dibromobutane (50 ml) was added to the mixture and boiled until it was neutral (approx. 3 hours). Ethanol was evaporated under reduced pressure and the residue was diluted with 30 ml of water and extracted with three 50 ml portions of ether.

The combined ethereal extracts were dried and evaporated. The unreacted 1,4-dibromobutane was evaporated *in vacuo*. The brownish residue was chromatographed with light petroleum on an alumina column (3 × 10 cm, neutral, act. II) until all halogen containing material was eluted (remaining 1,4-dibromobutane). Elution was continued with 100 ml of benzene and the eluate was evaporated to dryness. The residual oil was dissolved in a small amount of ether and diluted with light petroleum to incipient turbidity. Upon standing in a refrigerator at -10° C 3.85 g of diethyl ω-bromobutylacetamidomalonate crystallized out. Yield 17 %. Twice crystallized sample (ether-light petroleum) had m. p. 62° C. For $C_{13}H_{22}BrNO_5$ calculated : 3.98 % N; 22.70 % Br; found : 3.83 % N, 23.46 % Br.

Diethyl 2-acetamido-2-(5'-bromopentyl)malonate (IIb).

It was prepared in a manner analogous to II*a* from 9.8 g of diethyl acetamidomalonate and 50 ml of 1,5-dibromopentane. After purification on alumina and crystallization from ether-light petroleum, 3.28 g (14 %) of colourless crystals were obtained which after recrystallization from the same solvent mixture had m. p. 46° C. For $C_{14}H_{24}BrNO_5$ calculated : 3.82 % N, 21.83 % Br; found : 3.69 % N, 21.54 % Br.

Diethyl 2-acetamido-2-(6'-bromohexyl)malonate (IIc).

It was prepared similarly as II*a* from 9.8 g of diethyl acetamidomalonate and 50 ml of 1,6-dibromohexane. After purification on alumina and crystallization from ether-light petroleum 2.5 (11 %) of colourless crystalline products were obtained, m. p. 38° C. Double crystallization from the same solvent mixture gave a product melting at 41° C. For $C_{15}H_{26}BrNO_5$ calculated : 3.68 % N, 21.03 % Br; found : 3.67 % N, 20.39 % Br.

Tetraethyl 1,6-diacetamino-1,1,6,6-tetracarboxylate (IIIa).

Diethyl acetamidomalonate (10.8 g; 50 mmole) was added to a solution of 1.15 g of sodium (50 gram-atom) in 40 ml of abs. ethanol and boiled shortly. 1,4-Dibromobutane (5.5 g; 3.2 ml; 25 mmole) was added to the mixture and refluxed for approx. 7 hours (until neutral). The mixture was evaporated to dryness in vacuo, the residue was mixed with 20 ml water and extracted three times with 50 ml ethyl acetate. The combined extracts were dried and evaporated to dryness. The residue was dissolved in a minimum amount of boiling ether and light petroleum was added to incipient turbidity. The separated crystals were filtered off and crystallized from the same solvent mixture. Yield 2.5 g (20 %), m. p. 135° C. For $C_{22}H_{36}N_2O_{10}$ calculated : 54.14 % C, 7.74 % H, 5.74 % N; found : 53.76 % C, 7.34 % H, 5.77 % N.

Tetraethyl 1,7-diacetaminoheptane-1,1,7,7,tetracarboxylate (IIIb).

Using the same procedure as above *IIIb* was prepared from 10.9 g of diethyl acetamidomalonate and 6 g (3.5 ml) of 1,5-dibromopentane. Yield 2 g (17 %), m. p. 144° C. For $C_{23}H_{38}N_2O_{10}$ calculated : 55.02 % C, 7.63 % H, 5.58 % N; found 54.96 % C, 7.70 % H, 5.50 % N.

Tetraethyl 1,8-diacetaminooctane-1,1,8,8-tetracarboxylate (IIIc).

Dry sodium salt of diethyl acetamidomalonate was prepared from 10.9 g (50 mmole) of diethyl acetamidomalonate and 1.15 g of Na (50 gram-atom) in 45 ml of abs. ethanol. To the well ground sodium salt 50 ml of 1,6-dibromohexane were added and the mixture was heated at 140-150° C under thorough stirring for approximately 7 hours, i.e. until the mixture became neutral. After cooling the separated bromide was filtered off and the filtrate distilled at 15 mm Hg to remove excess dibromohexane. The brown residue was mixed with 30 ml of water and extracted with three 50 ml portions of ethyl acetate. The combined extracts were dried over magnesium sulfate and ethyl acetate was then evaporated. The oily residue crystallized after 24 hours standing. The crystals were triturated with ether, filtered, and crystallized twice from benzene-light petroleum. Yield 0.6 g (6.5 %), m. p. 137° C. For $C_{24}H_{40}N_2O_{10}$ calculated : 55.8 % C, 7.81 % H, 5.43 % N; found : 54.8 % C, 7.48 % H, 5.36 % N.

D,L-2,7-Diaminosuberic Acid-(2-^{14}C) (IVa).

Diethyl acetamidomalonate-(2-^{14}C) (218 mg; 1 mmole) of 17 μCi activity was added to 1 ml of 1-molar sodium ethoxide in ethanol and the mixture was boiled for a short period. The mixture was additioned with 0.35 g (1 mmole) of diethyl 2-acetamido-2-(4′-bromobutyl)malonate and boiled in an inert atmosphere, increasing the bath temperature and distilling off slowly the liberated alcohol. After 2 hours the bath temperature attained 160° C and the reaction mixture was kept at this temperature for another 4 hours. The cooled reaction mixture was decomposed with 1 ml of water and extracted continually with ether in a small liquid-extractor for 5 hours. The ethereal extract was evaporated and the white solid residue was hydrolysed under reflux with 6 ml of hydrochloric acid-acetic acid mixture (2 : 1) for 5 hours. The acids were evaporated *in vacuo* and the residual acidity was eliminated by double addition of distilled water and evaporation. The aqueous solution was decolorized with charcoal, evaporated to dryness, dissolved in a minimum amount of hot conc. ammonia, and boiled until all ammonia escaped and the volume was reduced to approx. 2 ml. Ethanol (2 ml) was added to the residue and the mixture was allowed to stand overnight. The crystalline acid was filtered off under suction and recrystallized once more in the same manner. The yield of the chromatographically pure diaminosuberic acid formed was 105 mg (52 %), total activity 8.8 μCi. Specific activity 17 μCi/mmole, m. p. over 360° C (browning).

D,L-2,8-Diaminoazelaic Acid-(2-^{14}C) (IVb).

This was prepared analogously as IV*a* from 218 mg of diethyl acetamidomalonate-(2-^{14}C) (act. 17 μCi) and 0.37 g of diethyl 2-acetamido-2-(5′-bromopentyl)malonate. Yield 140 mg (64 %) of a chromatographically pure substance, total activity 11.3 μCi, spec. activity 17 μCi/mmole, m. p. over 360° C (browning).

D,L-2,9-Diaminosebacic Acid-(2-^{14}C) (IVc).

This was prepared analogously as IV*a* from 218 mg of diethyl acetamidomalonate-(2-^{14}C) (total activity 17 μCi) and 0.38 g of diethyl 2-acetamido-2-(6′-bromohexyl)malonate. Yield 180 mg (78 %) of a chromatographically pure product, total activity 13 μCi, spec. activity 17 μCi/mmole, m. p. over 360° C (browning).

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