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Synthesis of N^{π}- and N^{τ}-(2-Hydroxyethyl)-L-histidines

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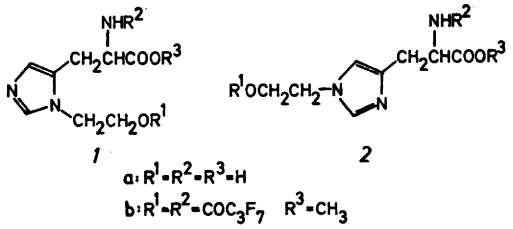
Treatment of N^{α}-acetyl-L-histidine methyl ester with ethylene oxide in methanol in the presence of hydrogen chloride and hydrolysis of the products gave N^{π}- and N^{τ}-(2-hydroxyethyl)-L-histidine in about equal amounts. Structural assignments of the isomers were based largely on the fragmentation pattern on electron impact of their bis(heptafluorobutyryl) derivatives. The acids are further characterized by their pK' values, specific rotation and chromatographic behaviour.

In this laboratory, studies are in progress aiming at the development of a chemical dosimetry that can serve as a basis for risk estimates in case of exposure to alkylating agents or to compounds metabolically transformed to such agents. Haemoglobin has been chosen as a suitable target molecule because of its long lifetime *in vivo* and its content of reactive histidine and cysteine sites.[1] Special attention has been given to exposure to hydroxyethylating agents such as ethylene oxide [2] or ethylene, after its metabolic conversion to the epoxide *in vivo*.[3] Furthermore, haemoglobin from mice treated with [^{14}C]-labelled vinyl chloride after reduction with sodium borohydride and hydrolysis, has been found to give, *i.a.*, 2-hydroxyethylated histidines.[4] In connection with these studies, ring-alkylated 2-hydroxyethyl histidines were required as reference compounds.[1,3,4] In part of the work, quantitation of the 2-hydroxyethyl histidines was performed by means of mass spectrometry using deuterium-labelled 2-hydroxyethyl histidine as an internal standard for multiple-ion detection.[2]

It has been shown that the *tele*-position * of the unprotonated imidazole ring of histidine is more reactive than the *pros*-position towards alkylating agents.[5–7] Also in the presence of hydrochloric acid, the imidazole nitrogens of histidine are reactive towards ethylene oxide, readily forming imidazolium derivatives.[8] In this case, alkylation of the ring nitrogens probably proceeds by a mechanism analogous to that of the acid-catalysed hydroxyethylation of pyridine.[9] In order to achieve comparable yields of N^{π}- and N^{τ}-2-hydroxyethylated derivatives, N^{α}-acetyl-L-histidine methyl ester was treated in hydrogen chloride in methanol with a large excess of ethylene oxide or tetradeuterioethylene oxide, respectively. Under these conditions, the interaction between the α-amino function and the protonated imidazole ring should be negligible, serving to counteract the unfavourable intrinsic reactivity of the *pros- versus* that of the *tele*-nitrogen.

The course of the reaction was followed by TLC of hydrolyzed samples of the reaction products. Three new amino acids, all negative to the Pauly-test [10] and thus apparently alkylated in the imidazole nucleus, were evident. One of these accumulated when the reaction time was prolonged. Moreover, it was more firmly retained than the other acids when the reaction products were applied to an ammonium-loaded cation exchange column at high pH values. For these reasons it was



* The nomenclature is in accord with the recommendations of IUPAC-IUBS (*J. Biol. Chem. 247* (1972) 977). The imidazole nitrogen proximal to the alanine residue of histidine is called the *pros*-nitrogen (symbol π) whereas the imidazole nitrogen farthest from the alanine residue is called the *tele*-nitrogen (symbol τ).

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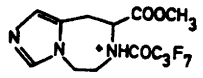
assumed to be the imidazolium-derivative N^{π},N^{τ}-di(2-hydroxyethyl)-L-histidine. In a final step, the amino acids not retained on the above column were separated on a similar H^+-loaded column eluted with 2 M HCl. N^{π}-(2-hydroxyethyl)-L-histidine (*1a*) and N^{τ}-(2-hydroxyethyl-L-histidine (*2a*) were formed in the ratio 9:10.

1a and *2a* were clearly distinguished by differences in the mass spectra of their respective derivatives *1b* and *2b*. Both derivatives showed the expected molecular ions and gave several identical fragments. One of the derivatives, however, gave a base peak, $m/e = 392$ ($m/e = 396$ from the deuterated analogue), completely absent in the mass spectrum of the other. The bicyclic fragment *3*, most probably responsible for this peak, can obviously only be formed from *1b*.



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Additional evidence for the proposed structures was provided by the IR spectra of the free acids. The spectrum of the acid, formulated as *1a*, displayed an absorption band at 12 μm, which was absent in the case of its isomer, in accordance with observations [11] on spectra of isomers of ring-alkylated histidines.

Further differences observed in the physical properties of the two isomers are also compatible with the structures discussed. These include:

(a) Pronounced tailing on GLC columns is exhibited by *1b* as compared to *2b*, the difference in tailing properties most probably being due to stronger interactions of the free *tele*-nitrogen with the stationary phase. The free *pros*-nitrogen of *2b* should be sterically more hindered.

(b) The acid formulated as *2a* is markedly stronger ($pK' = 5.45$) than its isomer (*1a*) which had $pK' = 6.1$. This difference can partly be attributed to the electron withdrawing effect of the protonated α-amino group which increases the acid strength of the protonated *pros*-nitrogen of *2a* more than that of the more distantly situated *tele*-nitrogen of *1a*.[12] Furthermore, the unprotonated *pros*-nitrogen of *2a* should have a higher tendency than the *tele*-nitrogen of *1a* to engage in intramolecular hydrogen bonding with the protonated α-amino group, thus forming a six-membered ring.[13] The difference in pK' values of the two isomers is analogous to that between N^{τ}-methyl-L-histidine ($pK' = 6.1$) [14] and N^{π}-methyl-L-histidine ($pK' = 6.58$).[15]

EXPERIMENTAL

Melting points were determined on a Kofler micro hot stage and specific rotations with a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Beckman 24 Spectrophotometer, IR spectra on a Perkin-Elmer 257 Grating infrared Spectrometer (KBr Technique) and mass spectra on an HP 5930A GC-MS quadrupole instrument connected to an HP 5933A computer system. pH values were measured on a pH-meter (Titrator TTT2, Radiometer) and the pK' values determined graphically by plotting ΔV NaOH/ΔpH as a function of pH. Elemental analyses were performed at the Microanalytical Laboratory, Ultuna, Sweden. The amino acid analyses were done at the Central Amino Acid Analysis Laboratory, Uppsala, Sweden. Unless otherwise stated the chemicals used were of analytical quality.

N^{α}-Acetyl-L-histidine methyl ester was prepared by dissolving N^{α}-acetyl-L-histidine [16] (5.1 mmol) in 1.25 M HCl in methanol (8 ml). The esterification was practically complete after 10 h at room temperature.

Hydroxyethylation of N^{α}-acetyl-L-histidine methyl ester was carried out in the above solution by the addition of ethylene oxide (125 mmol, Fluka). The solution was allowed to stand at room temperature for 24 h in a Pyrex tube equipped with a Teflon-lined screw-cap, and was then filtered and evaporated to dryness *in vacuo* at 40 °C.

Hydrolysis of the crude hydroxyethylated reaction product. The above reaction mixture (1.7 g) was dissolved in 2 M HCl (50 ml), and refluxed in an oil bath for 2 h. The hydrolysate was evaporated to dryness *in vacuo* at 75 °C (yield, 1.29 g).

Separation of the hydroxyethylated histidines required two steps, as *1a* and N^{π},N^{τ}-di(2-hydroxyethyl)-L-histidine eluted simultaneously on the Dowex 50 column when 2 M HCl was used as an eluent.

(a) *Separation on an NH_4^+-loaded ion-exchange column* was performed by applying the hydrolysate to a Dowex 50W-X4 (200–400 mesh, 43 × 2.1 cm) column, which was eluted with 13 mM NH_3, pH = 10.5. The eluate was collected in 15 ml fractions and the separation was followed by TLC on two stationary phases, cellulose and silica gel, developed with chloroform – methanol – ammonia 40:40:20. The R_F values were 0.63, 0.68, 0.68 and 0.53 on cellulose and 0.71, 0.74, 0.71 and 0.21 on silica

gel for L-histidine, *1a*, *2a* and N^{π},N^{τ}-di(2-hydroxyethyl)-L-histidine, respectively. In fractions 31 – 132, *1a* and *2a* were eluted, together with L-histidine, but free from the quaternary N^{π},N^{τ}-di(2-hydroxyethyl)-L-histidine. The latter was retained more firmly on the column and eluted in fractions 133 – 150. The concentration of NH_3 was critical for the separation, bad resolution being obtained with 130 or 1.3 mM NH_3.

(b) *Separation on an H⁺-loaded ion-exchange column.* The combined fractions 31 – 132 from the preceding separation were evaporated to dryness and applied to a Dowex 50W-X4 (71 × 4.6 cm) column, which was then eluted with 2 M HCl.

The first 4000 ml were discarded, after which 15 ml fractions were collected. The elution volumes of *1a*, *2a* and L-histidine were 5585 ml, 6355 ml and 6860 ml, respectively. The appropriate fractions were pooled and evaporated to dryness. The hydroxyethylated histidine hydrochlorides were transferred to the corresponding free amino acids by applying them to Dowex 50W-X4 (3 × 2.5 cm, H^+-form) columns which were eluted with water (100 ml), followed by 2 M NH_3 (50 ml). The ammoniacal eluates were evaporated to dryness (75 °C) *in vacuo*. The amino acids, obtained as syrups, were dried for 24 h in a vacuum desiccator.

The syrupy *1a* and *2a* were dissolved in methanol (5 ml) and crystallized overnight at 4 °C. The amino acids were recrystallized from methanol – 2-propanol.

N^{π}-*(2-Hydroxyethyl)*-L-*histidine (*1a*).* Yield: 0.65 mmol (13 %). Anal. $C_8H_{13}N_3O_3$: C, H, N. m.p. 222 – 224 °C (dec.). $[\alpha]_D^{22}$ −26.5° (H_2O). λ_{max}(H_2O, pH 7.0) 213 nm (ε = 5700). pK'_{im} (H_2O, 25 °C): 6.1. MS of N^2, *O*-bis(heptafluorobutyryl)-N^{π}-(2-hydroxyethyl)-L-histidine methyl ester [*m/e* (interpret.)]: 605 (M), 546 (M – CO_2CH_3), 392 (base peak, M – $C_3F_7CO_2$), 321 (M – $CH(CO_2CH_3)$ – $NHCOC_3F_7$).

N^{τ}-*(2-Hydroxyethyl)*-L-*histidine (*2a*).* Yield: 0.72 mmol (14 %). Anal. $C_8H_{13}N_3O_3$: C, H, N. m.p. 209 – 211 °C (dec.). $[\alpha]_D^{22}$ −20.8° (H_2O). λ_{max} (H_2O, pH 7.0) 209 nm (ε = 4600). pK'_{im} (H_2O, 25 °C): 5.45. MS of N^2, *O*-bis(heptafluorobutyryl)-N^{τ}-(2-hydroxyethyl)-L-histidine methyl ester [*m/e* (interpret.)]: 605 (M), 546 (M – CO_2CH_3), 321 (M – $CH(CO_2CH_3)$ – $NHCOC_3F_7$).

Hydroxyethylation of N^2-*acetyl*-L-*histidine methyl ester with ethylene oxide*-d_4. Tetradeuterioethylene oxide from Merck, Sharpe and Dohme Ltd., Montreal, Canada, was used. The reaction was performed in analogy with the procedure described for ethylene oxide, although on a scale which permitted characterization of the products only by mass spectrometry and by amino acid analysis.

N^{π}-*(2-Hydroxyethyl*-d_4*)*-L-*histidine.* MS of N^2,*O*-bis(heptafluorobutyryl)-N^{π}-(2-hydroxyethyl-d_4)-L-histidine methyl ester [*m/e*(interpret.)]: 609 (M), 550 (M – CO_2CH_3), 396 (M – $C_3F_7CO_2$), 325 (M – $CH(CO_2CH_3)$ – $NHCOC_3F_7$).

N^{τ}-*(2-Hydroxyethyl*-d_4*)*-L-*histidine.* MS of N^2,*O*-bis(heptafluorobutyryl)-N^{τ}-(2-hydroxyethyl-d_4)-L-histidine methyl ester [*m/e* (interpret.)]: 609 (M), 550 (M – CO_2CH_3), 325 (M – $CH(CO_2CH_3)$ – $NHCOC_3F_7$).

Chromatography

GLC required derivatization of the amino acids by means of 1.25 M HCl in methanol and heptafluorobutanoic anhydride.[2] The derivatives of *1a* and *2a* had retention times of 2.4 and 2.9 min, respectively. The conditions were: Column: 3 % SE-30 on Chromosorb W (AW, DMCS, 100 – 120 mesh), 1.8 m × 6 mm, 220 °C. Injection: 3 ng of each derivative, 265 °C. Detection: EC-^{63}Ni.

1b displayed tailing on the column and the peak shape impaired substantially when the injected amount was decreased. Moreover, the retention time increased as the injected amount was decreased (the retention time was 3.7 min for 0.5 ng).

Amino acid analyses were run on a Durrum D-500 amino acid analyzer. The retention times of *1a*, *2a* and L-histidine were 58.0, 57.3 and 58.5 min, respectively. The relative colour constants (valine = 1.00) of the amino acids were 0.92, 0.95 and 0.90, respectively. The analyses showed the preparations to be devoid of other ninhydrin-positive compounds.

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