An Investigation into the Catalytic Deuteration and Tritiation of Dehydroproline^{9}-Buserelin

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Summary

For optimizing the catalytic tritiation of peptides, the catalytic deuteration of buserelin and DhP^{9}-buserelin[1] in DMA and D_2O was studied by FAB-MS. The deuteration degrees achieved after reaction of DhP^{9}-buserelin were comparable in both solvents. The nonspecific incorporation of deuterium found after deuteration of buserelin was clearly lower after reaction in DMA. Tritiation of DhP^{9}-buserelin in DMA using 85% tritium gas, with conditions optimized by the deuteration experiments, resulted in a specific radioactivity of 1.3 TBq/mmol. 72 and 13 percent of the incorporated radioactivity were found to be associated with proline and histidine, respectively, after acidic hydrolysis.

Key words: peptide tritiation, solvent influence, nonspecific labelling

[1] Abbreviations: ACN, acetonitrile; BHA, benzhydrylamine; BOC, t.-butyloxycarbonyl; DCM, dichloromethane; DhP, L-3,4-dehydroproline; DIEA, N,N-diisopropylethylamine; DIPCDI, diisopropylcarbodiimide; DMA, dimethylacetamide; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenztriazole; TBPipU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-bispentamethyleneuroniumtetrafluoroborate.

Introduction

For biopharmaceutical studies the tritiated GnRH-analogue buserelin (pGlu-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-ethylamide) had to be synthesized.

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With regard to previous experiences in our group on the tritiation of GnRH-peptides (1) dehydroproline was chosen as an appropriate amino acid precursor for use in the present work. To find out the optimum conditions, deuteration experiments were performed with DhP-buserelin prior to the tritiation experiment and were monitored by FAB-MS.
At the same time these experiments offered an opportunity to compare with the results obtained from earlier model deuterations using a simple dehydroproline derivative (2), and to prove the validity of these results for the deuteration of a peptide. Additional experiments were performed to check the suitability of D_2O as an alternative to DMA in tritiations. Previous model deuterations showed clearly higher deuterium incorporation after reaction in DMA as compared with water (2-4). However, in peptide tritiations with DMA solubility problems and poor reaction rates were sometimes encountered. D_2O, with its higher bond strength compared to water and therefore a lower ability for the unwanted transfer of its hydrogen isotope, could serve as a compromise between DMA and water. Systematic studies on the suitability of D_2O as a solvent for tritiations are lacking.

Results and discussion

DhP^9-buserelin was prepared by solid phase peptide synthesis using a combination of Boc-/Fmoc-chemistry, base labile glycolamidic linkage (5-7) and minimal side-chain protection strategy (8). Based on this strategy other final deprotection/ cleavage procedures additionally to the aminolysis with ethylamine could be avoided.

The deuteration of DhP^9-buserelin resulted in a clearly higher degree of deuteration after reaction in DMA as compared with water (table 1), which is in accordance with earlier results obtained with N-acetyl-dehydroproline amide (2).
In contrast to the reaction of the simple amino acid derivative, which proceeded faster in water (2), the reaction rate for the deuteration of the peptide was higher in DMA . So, using a catalyst to substrate ratio of 0.5 mg catalyst/0.3 mg peptide, which was to low for a quantitative reaction, 60 and 73% of the starting compound were detected after 45 min deuteration in water and D_2O, respectively, by FAB-MS, but only 29% after reaction in DMA.

Based on these deuteration results DMA was used as the solvent for the tritiation of DhP-buserelin.

Table 1: FAB-MS results after 45 min catalytic deuteration of DhP-buserelin and buserelin in the presence of Al_2O_3 10% in 1 ml of solvent

Peptide	solvent	$\frac{I_{corr.m/z} \cdot 100}{\sum I_{corr.m/z\ 1240-1244}}$ [a]					D/mol
		x=1240 ($^2H_0$)	x=1241 ($^2H_2$)	x=1242 ($^2H_3$)	x=1243 ($^2H_3$)	x=1244 ($^2H_4$)	
DhP-buserel.	H_2O [b]	10.6	38.0	48.6	2.8	-	1.44
"	D_2O [b]	6.8	13.5	46.6	22.6	10.5	2.17
"	DMA [c]	5.1	20.3	45.8	20.3	8.5	1.91
Buser.	DMA [c]	89.6	6.2	4.2	-	-	0.14
"	D_2O [c]	55.7	31.4	7.1	5.7	-	0.63

[a] $I_{corr.m/z}$ = Intensity of the ms-peak appearing at the mentioned mass number diminished by the intensities of the corresponding natural abundance peaks related to x-1 - x-4
[b] 0.1 mg peptide/2 mg catalyst
[c] 1.0 mg peptide/5 mg catalyst

The tritiation was performed at a catalyst to substrate ratio determined by the deuteration experiments to guarantee a quantitative saturation of the double bond and yielded ^3H-buserelin with a specific radioactivity of 1.3 TBq/mmol (see Experimental).

This labelling result seems to be very low in comparison to the corresponding degree of deuteration shown in table 1. However, taking into account the hydrogen content of about 15% in the tritium gas and an isotopic effect of 5 as derived for the deuteration of N-acetyl-dehydroproline amide (2), modified for ^3H in analogy to (13) by an exponential factor of 1.4, both results correspond well.

Using D_2O as the solvent a deuteration result similiar to that in DMA was obtained (table 1). But this result is of only limited value for an assessment of the suitability of D_2O as a

solvent for tritiations because of the unknown degree of the transfer of the solvent hydrogen isotope. Additional tracer experiments with dehydroproline, using diluted tritium gas (table 2), however, demonstrated, that actually comparable degrees of labelling can be achieved after reaction in DMA and D_2O. After reaction in D_2O a more than 30% higher specific radioactivity was found as compared with water. This result was combined with a reduced solvent radioactivity (table 2), indicating a clearly lowered transfer of the solvent hydrogen

Table 2: Specific radioactivity of ^3H-proline and the tritium activity in the solvent obtained after catalytic tritiation of L-3,4-dehydroproline tartrate (1.3 mg) in the presence of Pd/Al_2O_3 10% (4 mg) using 50% tritium gas

Solvent	specific radioactivity of ^3H-proline (GBq/mmol)	^3H-activity in the solvent (GBq)
H_2O	144	4.9
D_2O	194	4.1

isotope with respect to water. Taking into consideration the same order of the differences in the degrees of labelling found in this tritiation experiment (table 2) and after deuterations in DMA and water, respectively, (table 1, (2)), D_2O seems to be an appropriated substitute for DMA in catalytic tritiations.

The value of D_2O as a substitute for DMA is limited, however, by a reduced position specificity of the labelling compared to that achieved after reaction in DMA, as can be derived from the deuteration results shown in tables 1 and 3. This effect is due to an unspecific labelling proceeding generally in catalytic tritiations of precursor peptides additionally to the reaction of the precursor amino acid. Preferable targets are the activated hydrogens in the position 2 of histidine rings and in benzylic positions of aromatic amino acid residues (10-12).

Accordingly, after the tritiation of DhP^9-buserelin 13 and 1.2% of the label were found in histidine and tyrosine, respectively, and the major part of this radioactivity could be assigned to the position 2 of the histidine ring by exchange experiments performed in analogy to (13) (see Experimental).

The position specificity of the labelling inside of the dehydroproline moiety is also clearly lower after reaction in D_2O compared with DMA, as can be derived from the distribution pattern in the molpeak region of the mass spectra obtained after deuteration of N-acetyl-dehydroproline amide (table 3). Surprisingly, in spite of the considerable amount of deuterium incorporated unspecifically during the reaction in D_2O into the target molecule, as indicated by the intensity of the M+3 - M+5-peaks ($^2H_3$ - $^2H_5$), the total amount of incorporated deuterium does not exceed markedly the number of two deuterons required for the saturation of the double bond. Thus, the amount of this additionally incorporated label must be compensated by a corresponding incorporation of hydrogen which is indicated by the presence of the M^+ - and M+1 - peaks in the mass spectra (tables 3 and 1). An analogous observation was also made after deuteration of N-acetyl-diiodotyrosine in D_2O (4). An explanation of these findings seems to be possible on the basis of a mechanism for the transfer of solvent hydrogen during catalytic deuterations proposed previously (14). In analogy to this mechanism the hydrogen should be exchanged by the catalyst from activated positions of the substrate prior to the saturation of the double bond and afterwards transferred again into the substrate during the actual reaction.

Table 3: Mass spectrometric results obtained after catalytic deuteration of N-acetyl-3,4-dehydroproline amide (5 mg) in the presence of Pd/Al_2O_3 10% (5 mg) according to (2).

Solvent	$\frac{I_{corr.m/z} \cdot 100}{\sum I_{corr.m/z\ 156-161}}$ [a]						D/mol
	x=156 ($^2H_0$)	x=157 ($^2H_1$)	x=158 ($^2H_2$)	x=159 ($^2H_3$)	x=160 ($^2H_4$)	x=161 ($^2H_5$)	
DMA	4.3	21.3	60.5	13.5	0.4	-	1.84
D_2O	5.8	21.5	42.4	23.8	5.5	1.0	2.05

[a] see footnote a) of table 1

That a major precondition for this mechanism holds, the catalyst bound hydrogen isotope must be able to react with the substrate more quickly than to exchange into the gas phase, was demonstrated before for dehalodeuterations (14). With view to

the tritiation practice this interpretation suggests that position specific labelling cannot be achieved quantitatively in catalytic tritiations even by use of pure tritium gas and solvents containing no hydrogen.

Experimental

Materials and methods

Fmoc- and Boc-protected amino acids were purchased from Novabiochem, with exception of L-Boc-dehydro-3,4-Pro-OH (Bachem). The BHA-resin (UR-95; 2% divinylbenzene; 1 mequiv./g) was obtained from Wolfen (Germany). DMF, DCM, MeOH, diethylether, piperidine, bromoacetic acid, TFA and DIEA were purchased from Merck-Schuchardt. Ethylamine, DIPCDI and HOBt were obtained from Fluka. DCM was distilled from anhydrous Na_2CO_3 and kept over 4A molecular sieves before use. DMA and DMF were distilled and kept prior to use over 4A molecular sieves for at least 2 weeks. TBPipU was prepared according to Henklein et al. (15). Buserelin acetate was a gift from the Hoechst AG (Germany).
To allow comparison, Palladium on alumina (10%) catalyst (Engelhard, Hannover) was of the same batch as that used in foregoing peptide tritiations (16). Tritium gas (^1H-content about 15%, estimated by means of an ionization Chamber) was purchased from Techsnabexport (Russia) and stored in the form of uranium tritide.

Model deuterations were performed by using flowing deuterium gas (produced by electrolyzing D_2O containing 5% H_2SO_4) as described previously (2).

Preparative HPLC was performed on a PLRP-S A300-10 (25x250 mm)-column (Polymer Lab) using the eluents A) 0.1% TFA and B) 50% ACN/0.1% TFA (gradient from 30 to 80% B within 70 min.) at a flow rate of 10 ml/min.

Analytical HPLC characterization was carried out on a Vydac C18 (4.6x150 mm)-column with the eluents A) 5% ACN/0.07 M NaH_2PO_4, pH 3 and B) 50% ACN/0.07 m NaH_2PO_4 (gradient from 1 to 95% B within 40 min.) at a flow rate of 1 ml/min.

MS/FAB analysis was performed on a TSQ 700 spectrometer (Finnigan MAT) using a glycerol matrix and xenon at 8 keV. For estimation degree of deuteration the average intensities obtained after about 100 scans were used.

Amino acid analyses were performed after hydrolyzing the peptides in 20% hydrochloric acid, containing 2% thioglycolic acid, in evacuated sealed tubes at 110°C for 20 h on an amino acid analyzer T 339 (Microtechna, Prague). For estimation of the distribution of tritium about 0.5 MBq of the labelled peptide were hydrolyzed together with 200 nmol of the unlabelled peptide and the fractions of the individual amino acids were collected and measured by liquid scintillation counting.

Racemization investigations were performed after hydrolysis in 20% hydrochloric acid and derivatization to form the trifluoroacetylated methylester derivatives by means of a GC-MS system (HP 5890, series II; MSD HP 5971; Hewlett Packard) using the chiral stationary phase Lipodex E (Macherey & Nagel).

Peptide synthesis

pGlu-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-dehydro-3,4-Pro-O-CH_2-CO-BHA-resin

The synthesis of the nonapeptide resin was carried out manually starting with 0.3 g of BHA-resin. The NH_2-groups of the resin were acylated using bromoacetic anhydride as described previously (5-7). L-Boc-dehydro-3,4-Pro-OH was coupled as the cesium salt according to Gisin (17) to yield the glycolamidic ester linkage. For coupling of the remaining residues of the sequence, the following protected amino acids were used:Boc-Arg(HCl), Boc-Leu, Fmoc-D-Ser(tBu), Fmoc-Tyr, Fmoc-Ser, Fmoc-Trp, Fmoc-His(Fmoc) and pGlu.
For the stepwise synthesis two equivalents of Boc- or Fmoc-amino acid, TBPipU and DIEA in 1.5 ml of DMF/DCM (1/1) were added to the resin. The pH was adjusted to 5-6 with DIEA (4 equiv.) and the mixture was stirred for 30 min. With Boc-Arg(HCl)-OH coupling was performed with DIPCDI/HOBt in DMF for 2 hours. Couplings were monitored by the qualitative ninhydrin test (18) and repeated if necessary. After the incorporation of pGlu, the peptidyl-resin was washed subsequently with DMF, DCM, MeOH, DCM and diethylether and dried *in vacuo*. Yield 567 mg.

pGlu-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-dehydro-3,4-Pro-NHC_2H_5

The peptide resin was suspended in 10 ml DMF/$NH_2C_2H_5$ (1/1, v/v) and stirred overnight at ambient temperature. Then the resin was separated off by filtration and washed subsequently with MeOH (3x10 ml) and diethylether (3x10 ml). The combined filtrates were evaporated *in vacuo*, yielding 203 mg of a crude product, which proved by HPLC to contain two major components in comparable amounts.
The mixture was purified by preparative HPLC and the component showing a HPLC retention time identical with that of buserelin (analyt. HPLC: t_r 22.1 min) was isolated and proved to be the required product by FAB-MS, amino acid analysis and racemization investigation. Yield 9.9 mg. Purity 98 % as checked by analytical HPLC (220 nm). FAB-MS: 1237.6 (MH^+), calcd. 1236.4 (M).
The other component (analyt. HPLC: t_r 23.1 min) showed identical results by FAB-MS and amino acid analysis. Racemization investigation revealed that this component contained D-3,4-dehydroproline instead of the L-derivative.

Tritium labelling

^3H-buserelin

1 mg DhP^9-buserelin dissolved in 0.5 ml of DMA was tritiated in the presence of 5 mg of 10% Pd/Al_2O_3 as described previously (16) over a period of 45 minutes at ambient temperature and a pressure of about 35 kPa using 85% tritium gas. After removing the labile tritium, 1.3 GBq of a crude labelled product were obtained which proved, by tlc (n-butanol/acetic acid/ water 3/1/1; R_F 0.5), to be identical with buserelin. After HPLC (Nucleosil C18-column, 250x4 mm; 25% ACN/75% triethylammoniumformiate, 0.04 m, pH 3.2; 1 ml/min.) about 30% of this radioactivity could be assigned to buserelin (t_r 14.5 min.). Two-fold purification in 0.2 ml portions by HPLC yielded a product with a radiochemical purity of about 98%.

Pharmacokinetic studies performed with this product showed the radioactivity to be highly accumulated in the pituitary as opposed to other tissues, which speaks well for its biological activity (Berger H. et. al., to be published).

The specific activity was determined to be 1.3 TBq/mmol on the basis of the area of the HPLC-peak (220 nm) in comparison with that of the reference peptide.

After amino acid analysis 72% of the label could be assigned to proline, 13% to histidine and 1.2% to tyrosine. The amount of radioactivity in fractions corresponding to other amino acids were below 1%.

For estimation of the tritium activity bound in position 2 of the histidine ring according to (13), 50 kBq of the purified labelled peptide, 20 nmol buserelin and 1 ul triethylamine, dissolved in 2 ml of water, were heated at 60°C for 30 h (exchange halflife time for ^3H in position 2 of the histidine ring under these conditions: 5.5 h (13)). After 30 minutes and 30 hours about 2% and 10%, respectively, of the total radioactivity were measured in the condensed water, indicating that about 9% of the total tritium activity are bound in position 2 of the histidine ring (about 1% of the activity found after 30 min. in the condensed water should be to assign to volatile impurities).

^3H-proline

1.3 mg L-3.4-dehydroproline tartrate were dissolved in 0.5 ml H_2O or D_2O (table 2), containing 10 ul of triethylamine, and tritiated as described previously (16) at ambient temperature and a pressure of about 35 kPa for 30 min. in the presence of 4 mg of 10% Pd/Al_2O_3, using the same batch of tritium gas, containing about 50% ^1H, in both cases. The crude products obtained after removal of the labile tritium were purified by tlc (n-butanol/acetic acid/ethylacetate/water 1/1/1/1), resulting in a radiochemical purity higher than 95% (tlc).

For estimation of the specific activities, 1.0 ml of 0.1 M borate buffer, pH 9.2 and 0.2 ml of a solution of 2.4-dinitrofluorobenzene in ethanol (10 mg/ml) were added to about 10 MBq of the purified labelled amino acid dissolved in 1.2 ml of water . After heating this solution at 60 °C for 1 hour and addition of 50 ul of 20% hydrochloric acid, the absorbance was measured at 380 nm. In the same way, using 50 - 200 nmol of proline, a calibration line was obtained.

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