8. Synthesis and Properties of a 2-Diazohistidine Derivative: a New Photoactivatable Aromatic Amino-Acid Analog

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N^{α}[(*tert*-Butoxy)carbonyl]-2-diazo-L-histidine methyl ester **1** was synthesized starting from the corresponding L-histidine derivative. The physico-chemical properties of this new photoactivatable amino-acid derivative were established. The synthetic precursor of **1**, 2-amino-L-histidine derivative **3**, was best isolated and characterized as 2-amino-N^{α}-[(*tert*-butoxy)carbonyl]-N^{τ}-tosyl-L-histidine methyl ester (**4**). Selective deprotections of **4** (N^{α}-Boc, N^{τ}-Tos, COOMe) were achieved, thus allowing the use of the corresponding products in peptide synthesis. The optically active dipeptides **8** and **9** were synthesized by coupling 2-amino-N^{τ}-tosyl-L-histidine methyl ester (**5**) with *N*-[(*tert*-butoxy)carbonyl]-L-alanine and N^{α}-[(*tert*-butoxy)carbonyl]-N^{τ}-tosyl-L-histidine (**6**) with L-alanine methyl ester, respectively. The question of selective diazotization of a 2-aminohistidine residue in a synthetic peptide was studied using competitive diazotizations between 2-amino-1*H*-imidazole and several amino-acid derivatives susceptible to undergo nitrosylation. The results show that synthetic photoactivatable peptides incorporating a 2-diazohistidine residue might become useful photoaffinity probes.

Introduction. – The discovery of numerous peptides involved in the recognition and modulation of hormone or neuromediator receptors stimulated the design and the synthesis of peptide analogs in order to study ligand-receptor interactions. Among these probes, photoactivatable structures were conceived to label irreversibly the receptor at a target binding site by means of photoaffinity labelling experiments [1]. Two major results are expected from this approach, either to identify and characterize an unknown receptor or to define at a molecular level the ligand-receptor interactions.

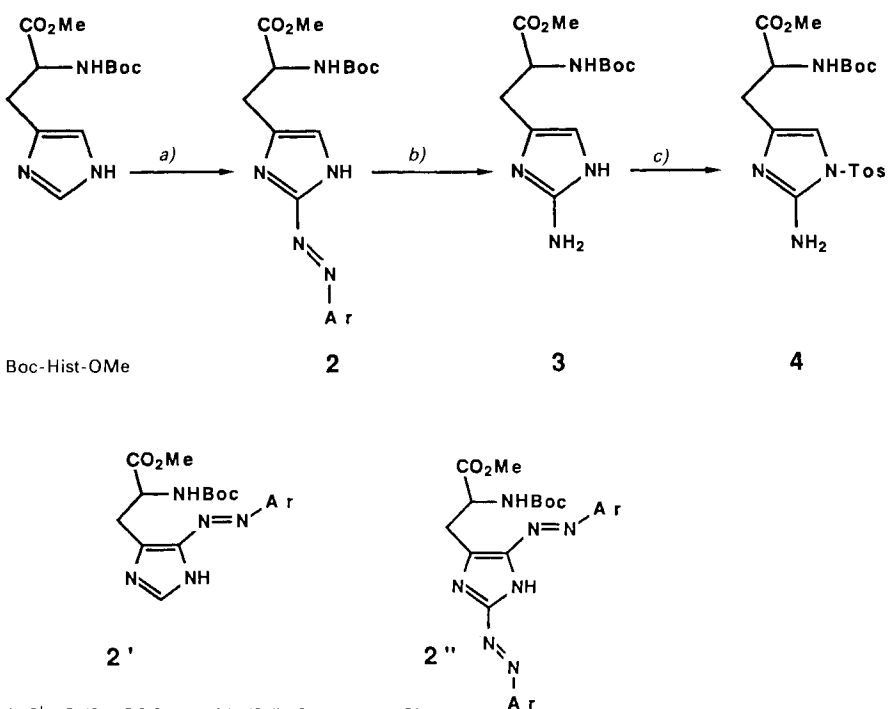
The synthesis of photoactivatable peptides was first conceived by tagging a photosensitive group to an existing peptide. Most of the described examples used electrophilic reagents bearing an arylazido or a benzophenone moiety, the coupling of the reagent to the peptide being generally directed towards the C-terminal [2] or towards nucleophilic residues existing on the peptide such as the primary amino group of a lysine side chain [3]. Besides the problem of selectivity of modification which can arise, the major limitation of this method is the consequent structural changes caused by the attachment of the photosensitive group on the natural ligand which often induces a loss in binding affinity. The design of a photosensitive amino-acid analog structurally related to a natural amino acid and which could be incorporated in the peptide during the synthesis (solid phase or in solution), would overcome the above mentioned limitations. As such, the 4′-azidophenylalanine derivative [4] has been developed and used as a substitute for phenylalanine in several peptides [5]. The syntheses of those peptides used a 4′-nitrophenylalanine precursor which was subsequently transformed to the corresponding 4′-azido derivative.

We report here the synthesis and the physico-chemical properties of a new photoactivatable amino-acid derivative, the N^{α}-[(*tert*-butoxy)carbonyl]-2-diazo-L-histidine methyl ester (Boc-Hist(2-N_2)-OMe; **1**). We also synthesized a fully protected 2-amino-L-histidine derivative, namely 2-amino-N^{α}-[(*tert*-butoxy)carbonyl]-N^{τ}-tosyl-L-histidine methyl ester (Boc-Hist(2-NH_2,τ-Tos)-OMe; **4**) as precursor of 2-diazo-L-histidine for peptide synthesis. Selective deprotection reactions of **4** and the potential use of the products in peptide synthesis are presented. The subsequent transformation of the 2-amino-L-histidine moiety to the corresponding 2-diazo derivative is also discussed.

Results and Discussion. – The synthesis of a peptide incorporating a photoactivatable amino-acid analog poses several problems such as the chemical stability of the photosensitive moiety and the necessity to achieve the synthesis in the dark. On the other hand, when using a precursor of the photosensitive group, stable during peptide synthesis, there is the question of selective transformation of this precursor on the peptide. We chose this second route by synthesizing the protected 2-amino-substituted L-histidine methyl ester **4**, a stable precursor of 2-diazo-L-histidine.

Synthesis of Boc-Hist(2-NH_2,τ-Tos)-OMe (**4**). The synthesis of **4** *(Scheme 1)* uses the known transformation of histidine derivatives to their corresponding 2-amino analogs, as described for an N^{α}-acetyl-L-histidine derivative [6]. However, the azo coupling reaction on the 2-position of the imidazole ring and the hydrogenolysis step both required substantial adaptation for the N^{α}-Boc series.

Scheme 1



a) 4-N_2^+–C_6H_4–COOMe. *b)* H_2/PtO_2. *c)* TosCl.

For the azo coupling with Boc-Hist-OMe, we tested a series of substituted diazonium salts $XArN_2^+$ (X = 4-Br, 4-SO_3H, 4-CO_2Me). Best results were obtained with the 4-methoxycarbonyl-substituted diazonium salt: satisfactory yields (65%) of pure 2-azo derivative **2** were achieved after separation (silica gel) from 5-azo isomer **2'** and 2,5-bisazo derivative **2''**. Comparable yields were obtained with the diazonium salt derived from sulfanilic acid, but the isolation and purification of the corresponding azo derivative proved more difficult.

Although a number of methods are known to cleave an azo linkage [7] (*b*) in *Scheme 1*), only hydrogenolysis of **2** in the presence of a great amount of PtO_2 (see *Exper. Part*) gave acceptable results. Again, the best results were obtained with the 4-methoxycarbonyl derivative: optimization of the reaction conditions (solvent, H_2 pressure, and amount of catalyst) led to 65% of pure 2-amino ester **3**. However, the presence of the 2-NH_2 group rendered **3** unstable which prompted us to synthesize its N^τ-tosyl derivative **4** by reaction with tosyl chloride at controlled pH. Noticeably, tosylation of **3** without prior isolation facilitated the purification procedures and gave improved yields of **4** (65% from **2**, 42% from Boc-Hist-OMe).

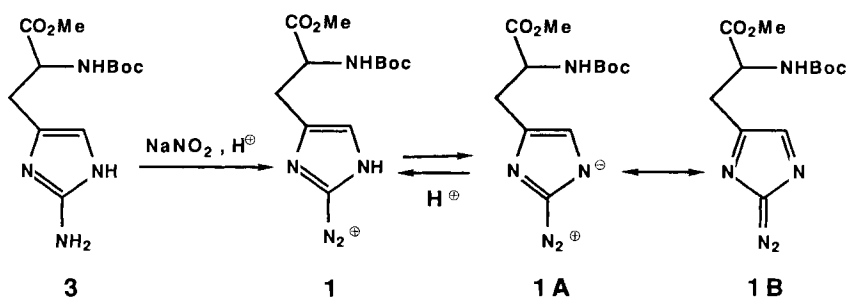
Diazotization of compound **4** led to the formation of a diazonium salt whose structure (NMR and UV; unpublished results) confirmed the position of the Tos group at N(1) (= N^τ) of the imidazole ring. On the other hand, the position of the side chain was tentatively attributed to C(4) of the ring based on steric-hindrance considerations and NMR spectroscopy [8].

The observed ^{1}H-NMR chemical shift (CD_3OD) of the imidazole proton of **4** (6.74 ppm) agrees with an H–C(5) rather than an H–C(4) for which a lower-field signal is expected, as deduced from the chemical shifts of the corresponding proton(s) of related imidazole derivatives (in CD_3OD); 2-amino-1*H*-imidazole: H–C(4)/H–C(5) at 6.77 ppm; 2-amino-1-tosyl-1*H*-imidazole: H–C(4) at 6.52 and H–C(5) at 6.97 ppm; **3**: H–C(4)/H–C(5) at 6.33 ppm.

Synthesis and Properties of Boc-Hist(2-N_2)OMe (**1**). Diazotization of 2-aminohistidine derivative **3** in 2M HCl/$NaNO_2$/H_2O at −5° gave, after chromatography on *Biogel P2*, pure (HPLC, C_{18} reverse phase) diazonium salt **1** in 60% yield (UV analysis; *Scheme 2*). No deprotection of the Boc group had occurred and the reaction could be followed spectrophotometrically (strong absorbance at λ_{max} 321 nm).

The acido-basic equilibrium (diazonium *vs.* diazo) described for 2-diazoimidazoles [9] (pK_a *ca.* 2.5) [10] is also expected for the corresponding histidines. The NMR and UV spectra of **1** are hardly influenced by the state of protonation which might be attributed to

Scheme 2



the presence of a zwitterionic structure **1A** rather than a delocalized resonance form **1B** in basic medium *(Scheme 2)*. Similar conclusions, based on ^{15}N- and ^{13}C-NMR experiments, have been proposed for 2-diazoimidazole-4,5-dicarbonitrile [11].

Table 1 summarizes the half-lives of **1** in different buffered solutions. As expected, the stability of **1** decreases with increasing pH. However, the nature of the buffer plays also an important role. The use of phosphate buffer seems to be appropriate for further photoaffinity experiments.

Table 1. *Half-Life of Boc-Hist(2-N_2)-OMe* (**1**) *in Different Buffers, Measured by the Disappearance of the Absorption at 321 nm*

Buffer	pH (temp.)	Half-life [h]
$K_1K_2PO_4$ (50 mM)	7.2 (20°)	60
Tris-HCl (40 mM)	8.0 (20°)	< 0.1
Tris-HCl (20 mM)	6.8 (20°)	0.8
Tris-maleate (50 mM)	6.5 (10°)	2.3

The chemical stability of a diazo function in peptides might be hampered by the neighborhood of amino acids having nucleophilic side chains. Thus we determined the half-life of 2-diazo-1*H*-imidazole, a model compound for 2-diazo-L-histidine derivatives, in the presence of a large excess (250-fold) of several nucleophilic amino acids *(Table 2)*. From the instantaneous reaction observed with the L-cystein derivative, it appears that the concomitant presence of L-cystein and 2-diazo-L-histidine residues on a peptide should be avoided. On the other hand, the much slower reaction observed with the L-lysine derivative would not exclude the presence of this amino acid in the peptide, unless a very favorable intramolecular cross-reaction occurs. All other amino acids tested showed very weak reactivity.

Table 2. *Half-life of 2-Diazo-1*H*-imidazole* ($4 \cdot 10^{-5}$ M) *in the Presence of Different Amino Acids in Citric Acid Phosphate Buffer (pH 7.2), Measured by the Disappearance of the Absorption Band at 325 nm*

Amino acid (10^{-2} M)	Half-life [h]
None	> 200
Boc-His-OH	30
Z-Tyr-OEt	30
Boc-Met-OH	92
Boc-Lys-OMe	1
NH_2-Cys-OMe	instant. reaction
NH_2-Cys-OMe (10^{-4} M)	instant. reaction

The photodecomposition of **1** in phosphate buffer (pH 7.2) led, as expected, to complete disappearance of the high absorption *(Fig.)*. The presence of an isobestic point is in agreement with the formation of a unique photodecomposition product, presumably the corresponding imidazolone derivative. Irradiation of 2-diazo-1*H*-imidazole in MeOH gave 2-methoxy-1*H*-imidazole as a unique compound, as determined by NMR (unpublished results).

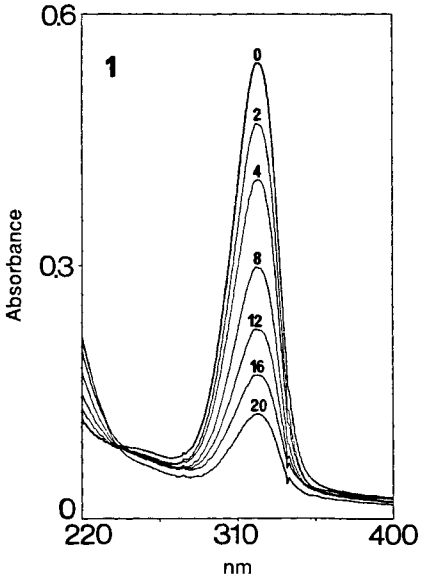
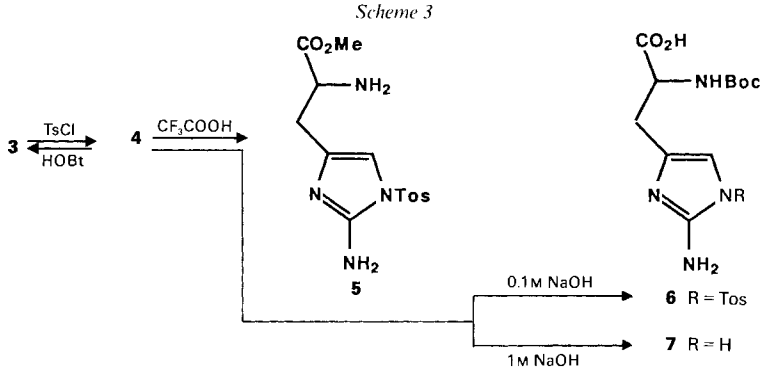


Fig. *Photodecomposition* ($\lambda_{irr.}$ 345 nm) *of Boc-Hist(2-N$_2$)-OMe* (**1**) *in* $K_1K_2PO_4$ (50 mM) *buffer* (pH 7.2). Time in min.

Selective Deprotections of Boc-Hist(2-NH$_2$-τ-Tos)-OMe (**4**; *Scheme 3*). The use of the protected 2-amino-L-histidine derivative in peptide synthesis requires a selective cleavage of either the N^{α}-amino or the carboxylic-acid protecting groups. The cleavage of the N^{α}-Boc protecting group was quantitatively achieved by CF_3COOH treatment in CH_2Cl_2, giving 2-amino-N^{τ}-tosyl-L-histidine methyl ester (**5**). On the other hand, mild basic treatment (0.1M NaOH) selectively hydrolysed the methyl-ester group yielding the N^{α}-Boc- and N^{τ}-Tos-protected amino acid **6**. Hydrolysis of both ester groups was achieved with 1M NaOH (→**7**). Like **3** the non-tosylated 2-amino-L-histidine derivative **7** was unstable and could not be characterized directly, but it was identical (TLC) with the compound obtained on saponification of methyl ester **3**.

Besides the Tos group, several other protecting groups for the 2-aminoimidazole moiety were considered. This group should be stable under the conditions used either for

Scheme 3

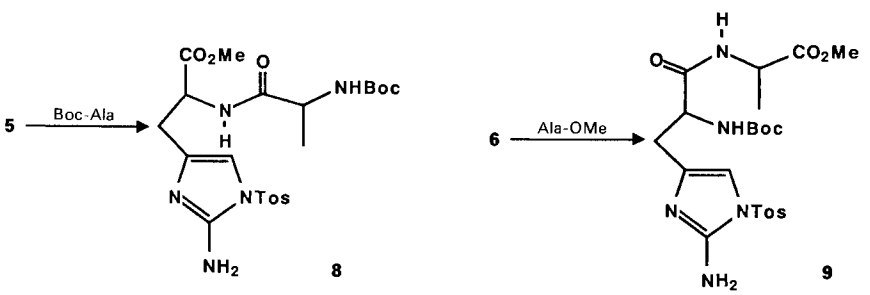


ester (COOMe hydrolysis) or carbamate cleavage at N(α) and, in addition, it should be easily removable. The 4-nitrophenyl moiety, a common imidazole-protecting group [12], could not be incorporated on the imidazole ring of **3** with satisfactory yields. The N^{τ}-benzyloxycarbonyl derivative synthesized by treatment of **3** with benzyl chloroformate appeared to be very sensitive under both acidic and basic conditions, *i.e.* deprotection already occurred in the presence of $NaHCO_3$ (unpublished results). On the contrary, the N^{τ}-tosyl moiety of **4** was stable during the acid treatment used for the N^{α}-Boc removal and it showed increased stability under basic conditions, allowing selective methyl-ester deprotection. During preliminary peptide coupling experiments using HOBt (benzotriazol-1-ol), the N^{τ}-Tos group was partially cleaved. Independent treatment of **4** with 2 equiv. of HOBt led to complete and selective cleavage of the N^{τ}-Tos group (→**3**), a result in agreement with [13].

The enantiomeric purity of the partially deprotected derivatives **5** and **6**, subsequently used for peptide synthesis, was checked by reaction with the optically active 2-fluoro-3,5-dinitrophenyl-L-alanine amide (FDAA, *Marfey's* reagent) [14]. The coupling of amino ester **5** with FDAA led to a 95:5 ratio of diastereoisomers after separation on HPLC. In the absence of reference samples, we used the derivatives of D- and L-histidine methyl ester obtained with *Marfey's* reagent to confirm that the D,L-diastereoisomer is eluted first. Similarly, 2-amino-N^{τ}-tosyl-L-histidine (obtained from **6** after CF_3COOH treatment) revealed a 96.5:3.5 ratio (HPLC) of the corresponding diastereoisomers. Again the HPLC elution profile (L,L-diastereoisomer first) corresponded to the one obtained with the FDAA derivatives of L- and D-histidine. An identical isomer ratio (96:4) was obtained for 2-amino-*N*-tosyl-L-histidine on coupling with the chiral fluorophore (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [15] (unpublished results). The weak racemization observed for both **5** and **6** might be attributed to the basic reaction conditions used during the azo coupling reaction *(Scheme 1, a)*.

Synthesis of Protected Dipeptides **8** *and* **9**. Peptides containing a 2-amino-L-histidine derivative have been described [16]; these syntheses were achieved by modifying the L-histidine residue on the presynthesized peptide using the azo coupling methodology. The cleavage of this azo linkage seems even more difficult when performed on a peptide instead of an amino acid, *i.e.* a 10% yield was achieved for the hydrogenolysis of a 2-azo-L-histidine derivative of TRH (Glp-His-ProNH$_2$) [16]. Moreover, this approach also implies that the chosen peptide does not contain other residues susceptible to azo coupling.

Scheme 4



The use of derivative **5** or **6** during peptide synthesis will avoid the aforementioned difficulties. To demonstrate this, we have synthesized dipeptides **8** and **9** from **5** and **6**, respectively *(Scheme 4)*. Reaction of **5** with the symmetrical anhydride of *N*-[(*tert*-butoxy)carbonyl]-L-alanine (prepared by dehydration of Boc-Ala in the presence of DCC) in CH_2Cl_2 containing Et_3N gave the expected dipeptide **8** in 59% yield after chromatography on silica gel and recrystallization. This yield is satisfactory, taking into account the non-optimized reaction conditions, *i.e.* 1 equiv. of each amino acid for the coupling. Dipeptide **9** was synthesized by reacting **6** with Ala-OMe in a biphasic medium (1,2-dichloroethane/H_2O) in the presence of a H_2O-soluble carbodiimide reagent [17]. Similar coupling yields of pure dipeptide **9** (57%) could be obtained after chromatography on silica.

Diazotization and Peptide-Synthesis Strategies. Theoretically, it will be possible to synthesize a fully protected peptide containing a 2-amino-N^{τ}-tosyl-L-histidine residue either from the N- to the C-terminal using side-chain protected α-amino esters, including **5**, or from the C- to the N-terminal using side-chain protected N^{α}-Boc-amino acids, including **6**. The reaction conditions used during the growth of the peptide (mild saponification conditions and acidic conditions) are compatible with the stability of the N^{τ}-Tos protective group. The purification of such fully protected peptides by HPLC can be envisaged [18]. Finally, selective removal of the N^{τ}-Tos group on the 2-amino-1*H*-imidazole ring (HOBt treatment) might allow one to direct the diazotization reaction exclusively to this moiety. To check this hypothesis, we tested the diazotization of 2-amino-1*H*-imidazole, a model compound for 2-amino-L-histidine, in competition with other amino-acid derivatives, in particular differently protected lysine and arginine derivatives *(Table 3)*. Thus, diazotization occurred exclusively at the 2-amino-1*H*-imidazole ring when fully protected competitors were used (*Table 3, cf. Entries 2* and *5* and *Entry 1*). On the other hand, diazotizations performed in the presence of lysine or arginine derivatives with α-amino group and side chain unprotected (*Entries 3* and *8*) allowed only a partial reaction on the imidazole ring (*ca.* 50%). Under those conditions, the use of 2.2 equiv. of $NaNO_2$, was required for the diazotization to go to completion (*Entries 4* and *9*), suggesting the consumption of 1 equiv. of $NaNO_2$ by another NH_2 group present. To determine which NH_2 moiety (α *vs.* ε) of the unprotected competitor interfers with

Table 3. *Competitive Diazotizations between 2-Amino-1*H*-imidazole* (**A**) *and Several Lys and Arg Derivatives at Equimolar Concentration*

Entry	Diazotization mixture in CF_3COOH	Equiv. of $NaNO_2$	Measured extinction coefficient ε (325 nm)[a])
1	**A**	1.1	19500 ± 700
2	**A** + Z-Arg(diZ)-OH	1.1	20800 ± 800
3	**A** + H-Arg-OMe	1.1	9700 ± 100
4	**A** + H-Arg-OMe	2.2	21400 ± 1400
5	**A** + Z-Lys(Z)-OH	1.1	19300 ± 1000
6	**A** + Z-Lys-OH	1.1	17400 ± 700
7	**A** + H-Lys(Z)-OH	1.1	22000 ± 2200
8	**A** + H-Lys-OH	1.1	11500 ± 500
9	**A** + H-Lys-OH	2.2	21900 ± 700

[a]) Mean value of three separate experiments.

2-amino-1*H*-imidazole during the diazotization, we tested the corresponding mono-protected lysine derivatives. Clearly, only the ε-NH_2 is competitive *(Entry 6)* while no influence of the α-NH_2 is observed *(Entry 7)*.

These results confirm the viability of the proposed synthetic strategy, *i.e.* synthesis and purification of a fully protected peptide incorporating the 2-amino-N^{τ}-tosyl-L-histidine residue followed by selective deprotection (HOBt) and diazotization on the target residue. The final step involves deprotection of all other protective groups without altering the 2-diazo-L-histidine residue. Anhydrous HF [19] or CF_3COOH/CF_3SO_3H [20] being often used as universal cleavage reagents, we checked that 2-diazo-1*H*-imidazole (in fact the corresponding diazonium salt) is stable under such conditions (unpublished results).

Conclusion. – The syntheses of 2-amino-L-histidine derivatives and N^{α}[(*tert*-butoxy)-carbonyl]-2-diazo-L-histidine methyl ester (**1**) are described. Compound **1** constitutes a new photosensitive α-amino-acid derivative for which the physico-chemical properties of a potential photoaffinity reagent were established. In particular, we determined the chemical stability of **1** in different buffers and in the presence of nucleophilic amino acids. The well established photosensitivity of diazo species was also confirmed by a total photo-destruction of **1**. The irradiation wavelengths employed (> 300 nm) are non-destructive for proteins and ideal for photoaffinity labelling experiments. The precise nature of the carbenes derived from 2-diazo-L-histidine derivatives are unknown. However, the carbene obtained from 2-diazo-1*H*-imidazole-4,5-dicarbonitrile had a highly electrophilic singlet character as illustrated by its ability to react with molecular N_2 [21]. From these studies, it can be concluded that 2-diazo-L-histidine derivatives are suitable probes for photoaffinity labelling experiments.

As suggested by the synthesis of dipeptides **8** and **9** *(Scheme 4)* and the results concerning the competitive diazotizations *(Table 3)*, more elaborate peptides containing a 2-amino-L-histidine residue could be synthesized by solid-phase methodology as long as the cleavage reaction from the polymer resin does not remove the N-terminal and side-chain protective groups. In the future, the diazotization step should be achieved on a fully protected peptide. This aspect of the problem has not been considered for the synthesis of peptides containing 4′-azido-L-phenylalanine derivatives [4] which also uses a final diazotization step. However, in that work an aromatic amine with lower pK_a values compared to the 2-amino-1*H*-imidazolium group (pK_a of 8.46 [22]) was transformed which might be responsible for selectivity of the diazotization.

The structure of 2-diazo-L-histidine is very similar to that of L-histidine. A structural analogy can also be claimed with L-arginine although 2-diazo-L-histidine, being a neutral amino acid, misses the positive charge found on the L-arginine side chain. The different possibilities of chemical and structural analogies are currently being investigated in enzymatic systems which are specific for these two amino acids. Our final goal is the synthesis of peptides in which an L-histidine, L-arginine, or even an L-phenylalanine residue is substituted by our new photoactivatable 2-diazo-L-histidine. The mentioned enzymatic studies should facilitate the judicious prediction of substitution.

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Experimental Part

General. HPLC: *Waters* automated gradient controller instrument. M.p.: calibrated *Kofler* hot-stage apparatus, uncorrected. UV spectra: *Uvikon 860* instrument; $\lambda_{max}(\varepsilon)$ in nm. NMR spectra: *Bruker WP SY 200* (200 MHz); Me_4Si as internal reference; chemical shifts in δ (ppm). MS: *LKB 9000S* instrument with EI source (70 eV, 100°) and *Thomson THW 208* instrument with CI source (NH_3, 100°). Elemental analyses (C, H, N) were performed by the Service de Microanalyses de l'Université Louis Pasteur, Strasbourg.

N^{α}-*[(*tert*-Butoxy)carbonyl]-2-diazo-*L*-histidine Methyl Ester* (**1**). Ester **3** (59 mg, 0.17 mmol) was dissolved in 2M aq. HCl (0.86 ml) at −5°. Precooled 2M aq. $NaNO_2$ (0.1 ml) was added to this well stirred soln. within 30 min. The crude mixture containing the diazonium salt (69% by UV) was chromatographed on *Biogel P₂* (*Biorad*, 200–400 mesh; column, 1.2 × 30 cm). The column was equilibrated with H_2O and eluted in the dark at 5° at a rate of 0.3 ml/min. The fractions were analyzed by UV and HPLC (C_{18} reverse phase, *Altex Analytic*, 5μ diameter): 60% of diazonium salt. Lyophilization of a sample of pure **1** gave a white powder for UV and NMR analysis. UV (H_2O): 321 (22000). ^{1}H-NMR (D_2O): 7.45 (*s*, 1 H); 4.70–4.50 (*m*, 1 H); 3.81 (*s*, 3 H); 3.34 (*dd*, *J* = 5, 15, 1 H); 3.17 (*dd*, *J* = 9, 15, 1 H); 1.43 (*s*, 9 H). ^{1}H-NMR (D_2O, DCl; diazonium salt): 7.36 (*s*, 1 H); 4.55–4.35 (*m*, 1 H); 3.68 (*s*, 3 H); 3.30 (*dd*, *J* = 5, 15, 1 H); 3.15 (*dd*, *J* = 9, 15, 1 H); 1.28 (*s*, 9 H).

N^{α}-*[(*tert*-Butoxy)carbonyl]-2-[4-(methoxycarbonyl)phenylazo]-*L*-histidine Methyl Ester* (**2**). Ice-cold 1.0M aq. $NaNO_2$ (10 ml) was added dropwise within ½ h to ice-cold and well stirred 2.3M aq. HCl (50 ml) containing methyl-4-aminobenzoate (1.51 g, 10 mmol). After 1 h additional stirring at 0°, the soln. containing the diazonium salt was added to a cold soln. of N^{α}-[(*tert*-butoxy)carbonyl]-L-histidine methyl ester (2.69 g, 10 mmol) 200 ml of a sodium tetraborate buffer prepared by adding an aq. soln. (150 ml) of $Na_2B_4O_7 \cdot 10\ H_2O$ (14.286 g) to 300 ml of 0.18N NaOH (final pH 10.5). During the addition (1 h), the pH was maintained at 9–9.5 by addition of 1N NaOH. After ½ h stirring at 0°, the suspension was neutralized (pH 7.4) with 5% HCl soln. and cooled for 10 h before filtration. The crude product was washed with H_2O and dried in a dessicator. Dissolved in a minimum of AcOEt, the crude product was adsorbed on SiO_2 and chromatographed on a silica-gel column (450 g; AcOEt/hexane 4:6). Elution with AcOEt/hexane 1:1 gave **2″**, followed by the desired **2** (AcOEt/hexane 6:4). After trituration in hexane, 2.8 g (65%) of **2**, were obtained as orange powder. **2:** M.p. 154°. ^{1}H-NMR ($CDCl_3$): 8.18 (*d*, *J* = 8, 2 H); 7.97 (*d*, *J* = 8, 2 H); 7.10 (*s*, 1 H); 4.70–4.60 (*m*, 1 H); 3.96 (*s*, 3 H); 3.77 (*s*, 3 H); 3.30–3.20 (*m*, 2 H); 1.44 (*s*, 9 H). CI-MS (CH_4): 432, (*M* + 1), 376 (100), 332 (24). Anal. calc. for $C_{20}H_{25}N_5O_6$: C 55.68, H 5.84, N 16.23; found: C 55.34, H 5.80, N 16.01.

2-Amino-N^{α}-*[(*tert*-butoxy)carbonyl]-*L*-histidine Methyl Ester* (**3**). PtO_2 (430 mg) was added to a soln. of **2** (431 mg, 1 mmol) in dry MeOH (20 ml) and submitted to hydrogenation (60 psi) for 20 h. After addition of AcOH (57 μl, 1 mmol), the catalyst was filtrated and washed with MeOH (3 × 10 ml). After evaporation without heating, the obtained powder was dissolved in H_2O (30 ml). After filtration, the aq. soln. was extracted thrice with 30 ml of Et_2O in order to eliminate the formed methyl 4-aminobenzoate and lyophilized: 223 mg (65%) of **3**·AcOH. ^{1}H-NMR (CD_3OD): 6.33 (*s*, 1 H); 4.33 (*dd*, *J* = 4, 8, 1 H); 3.72 (*s*, 3 H); 3.00–2.75 (*m*, 2 H); 1.44 (*s*, 9 H). MS: 284 (M^+), 228 (10), 211 (23), 167 (92), 96 (100).

2-Amino-N^{α}-*[(*tert*-butoxy)carbony]-*N^{τ}-*tosyl-*L*-histidine Methyl Ester* (**4**). A sat. aq. $NaHCO_3$ soln. was added at 0° to a aq. soln. of **3** (0.7 mmol in 20 ml), up to pH 8.7. At 0°, an acetone soln. (20 ml) containing 1.1 equiv. of TsCl (150 mg) was added dropwise during 1 h, maintaining the pH at 8.7 by addition of the $NaHCO_3$ soln. After 2 h stirring, more TsCl (0.5 equiv.) was added and allowed to react for 12 h at r.t. The obtained suspension was filtered off, the acetone evaporated, and the remaining aq. soln. extracted 4 times with 30 ml of CH_2Cl_2. The org. phase was washed with a sat. NaCl soln. (Na_2SO_4), and evaporated. The crude product was purified by flash chromatography (silica gel, AcOEt/hexane 6:4). The product was crystallized by trituration with Et_2O and hexane: 165 mg (53%) of **4**. M.p. 50°. $[\alpha]_D = -8.7$ (*c* = 9.09 · 10^{-3}, MeOH). ^{1}H-NMR ($CDCl_3$): 7.78 (*d*, *J* = 8, 2 H); 7.35 (*d*, *J* = 8, 2 H); 6.62 (*s*, 1 H); 5.62 (br., 1 H); 5.33 (br., 2 H); 4.60–4.40 (*m*, 1 H); 3.64 (*s*, 3 H); 2.87–2.75 (*m*, 2 H); 2.44 (*s*, 3 H); 1.43 (*s*, 9 H). CI-MS (NH_3): 439 (*M* + 1), 283 (53), 227 (52). Anal. calc. for $C_{19}H_{26}N_4O_6S$: C 52.04, H 5.98; found: C 52.20, H 6.13.

2-Amino-N^{τ}-*tosyl-*L*-histidine Methyl Ester* (**5**). For ½ h, **4** (50 mg) was treated with a 33% CF_3COOH soln. in CH_2Cl_2 (3 ml). After evaporation, the residue was recrystallized from Et_2O/hexane: 100% yield. M.p. 105°. $[\alpha]_D = -6.8$ (*c* = 8.1 · 10^{-3}, MeOH). ^{1}H-NMR (CD_3OD): 7.96 (*d*, *J* = 9, 2 H); 7.50 (*d*, *J* = 9, 2 H); 7.02 (*s*, 1 H); 4,27 (*d*,*d*, *J* = 6, 7.5, 1 H); 3.77 (*s*, 3 H); 3.03–2.95 (*m*, 2 H); 2.47 (*s*, 3 H). MS: 338 (M^+), 279 (63), 250 (89), 183 (21), 155 (100), 124 (84).

2-Amino-N^{α}-*[(*tert*-butoxy)carbonyl]-*N^{τ}-*tosyl-*L*-histidine* (**6**). To a soln. of 180 mg (0.41 mmol) of **4** in MeOH (4.5 ml), 1.1 equiv. of 0.1N NaOH (4.5 ml) were added dropwise at 0°, and after 2 h stirring, another 0.2 ml of 0.1N NaOH were added. After 1 h of additional stirring, the MeOH was evaporated and the aq. phase washed with Et_2O (elimination of remaining **4**), then acidified to pH 5 with AcOH, and finally extracted 4 times with 15 ml

of AcOEt. The org. phase was washed with sat. NaCl soln., dried (Na_2SO_4), and evaporated: 157 mg (90%) **6**. M.p. 157°. $[\alpha]_D = -7.0$ ($c = 9.55 \cdot 10^{-4}$, MeOH). ^{1}H-NMR ($CDCl_3$): 7.78 (*d*, $J = 8$, 2 H); 7.49 (br., 2 H); 7.39 (*d*, $J = 8$, 2 H); 6.51 (*s*, 1 H); 5.36 (br., 1 H); 4.40–4.20 (*m*, 1 H); 3.10–3.00 (*m*, 2 H); 2.47 (*s*, 3 H); 1.48 (*s*, 9 H). MS: 425 ($M + 1$), 253 (100), 153 (65).

Deprotection of **4** *with 1*N *NaOH.* The treatment of a cold (0°) MeOH soln. (0.4 ml) of **4** (22 mg) with 1N NaOH (0.2 ml) resulted in complete disappearance of **4** after 0.5 h. Anal. TLC (BuOH/AcOH/H_2O 25:4:10): single product, R_f 0.43.

The basic hydrolysis of **3** gave also a unique product with identical R_f, presumably *2-amino-*N$^{\alpha}$*-[(*tert*-butoxy)carbonyl-*L*-histidine]* (**7**). We did not try to isolate and characterize **7** which was relatively unstable.

Deprotection of **4** *with HOBt.* Benzotriazol-1-ol (27 mg, 0.2 mmol) was added as a solid to an MeOH (0.5 ml) soln. of **4** (44 mg, 0.1 mmol). The mixture was stirred for 1 h at r.t. before evaporation. The residue was taken in H_2O (5 ml) and washed 3 times with Et_2O (5 ml). Anal. TLC (BuOH/AcOH/H_2O 24:4:10) revealed that the org. phase contained mainly 1-(tosyloxy)benzotriazole (comparison with a synthetic sample [13]) as well as a small amount of unreacted **4**. The aq. phase contained excess HOBt, a small amount of 1-(tosyloxy)benzotriazole, and **3**. Lyophilization gave 37.3 mg of mixture which was quantified by NMR: HOBt/TsOBt/**3** 1:0.1:0.7, corresponding to 70% yield. **3**: ^{1}H-NMR ($DDCl_3$): 6.41 (*s*, 1 H); 5.31 (br., 1 H); 4.54 (br. 1 H); 3.69 (*s*, 3 H); 3.08–2.93 (*m*, 2 H); 1.32 (*s*, 9 H).

N$^{\alpha}$*-[(*tert*-Butoxy)carbonyl]-*L*-alanyl-2-amino-*N$^{\tau}$*-tosyl-*L*-histidine Methyl Ester (Boc-Ala-Hist(2-NH$_2$, τ-Tos)-OMe;* **8**). A suspension of the CF_3COOH salt of **5** (56.2 mg, 0.1 mmol) in CH_2Cl_2 (3 ml) was neutralized by the addition of 2 equiv. (28 µl) of (i-Pr)$_2$NH (→ homogeneous medium). Preparation of 1 equiv. of the symmetrical anhydride of Boc-Ala was performed by reacting Boc-Ala (37.8 mg, 0.2 mmol) in CH_2Cl_2 (2 ml) with a soln. of dicyclohexylcarbodiimide (DCC; 20.6 mg, 0.1 mmol) in CH_2Cl_2 (1 ml). After ½ h stirring at 25°, the mixture was cooled and the urea derivative filtered off. The filtrate was added to the neutralized soln. of **5** after 1 h stirring at 25°, the org. phase was washed thrice with 5 ml of H_2O and sat. NaCl soln., dried (Na_2SO_4), and evaporated. The crude product was purified by prep. TLC (CH_2Cl_2/MeOH 9.5:0.5). A large UV-visible band (R_f 0.6) was isolated and reeluted with CH_2Cl_2/EtOH 9:1. After evaporation of the solvent, the solid was recrystallized from CH_3CN/H_2O: 30 mg (59%) of **8** as a white powder. M.p. 145°. $[\alpha]_D = -32.8$ ($c = 8.2 \cdot 10^{-4}$, MeOH). ^{1}H-NMR ($CDCl_3$): 7.77 (*d*, $J = 8$, 2 H); 7.38 (*d*, $J = 8$, 2 H); 6.60 (*s*, 1 H); 5.77 (br. *s*, 1 H); 5.28 (br., 2 H); 4.69 (br. *s*, 1 H); 4.17 (*m*, 1 H); 3.72 (*q*, $J = 7$, 1 H); 3.61 (*s*, 3 H); 2.82 (*m*, 2 H); 2.43 (*s*, 3 H); 1.43 (*s*, 9 H); 1.34 (*d*, $J = 7$, 3 H). FAB-MS: 510 ($M + 1$), 454 (22), 356 (65).

*2-Amino-*N$^{\alpha}$*-[(*tert*-butoxy)carbonyl]-*N$^{\tau}$*-tosyl-*L*-histidyl-*L*-alanine Methyl Ester (Boc-Hist(2-NH$_2$, τ-Tos)-Ala-OME;* **9**). To a soln. of 50 mg (0.118 mmol) of **6**, 16.5 mg (1 equiv.) of H-Ala-OMe·HCl, and 16.58 µl (1 equiv.) of Et_3N dissolved in 1,2-dichloroethane (5 ml), an aq. soln. (200 µl) containing (ethyl)[3-(dimethylamino)-propyl]carbodiimide hydrochloride (22.62 mg, 1 equiv.) was added, and after 18 h stirring, 1 more equiv. of the H_2O-soluble carbodiimide was added. After 2 days, the starting compounds had completely disappeared. The org. phase was washed thrice with 2 ml of H_2O, dried (Na_2SO_4), and evaporated. The crude product was purified by prep. TLC (CH_2Cl_2/MeOH 9:1): 34.5 mg (57%) of **9**. M.p. 82°. $[\alpha]_D = -11.6$ ($c = 9.1 \cdot {}^{-5}$, MeOH). ^{1}H-NMR ($CDCl_3$): 7.80 (*d*, $J = 8$, 2 H); 7.34 (*d*, $J = 8$, 2 H); 6.63 (*s*, 1 H); 5.85 (br., 1 H); 5.41 (br., 2 H); 4.51–4.36 (*m*, 2 H); 3.72 (*s*, 3 H); 2.80–2.63 (*m*, 2 H); 2.44 (*s*, 3 H); 1.43 (*s*, 9 H); 1.24 (*d*, $J = 4$). FAB-MS: 510 ($M + 1$), 454 (19), 393 (15), 354 (14), 298 (15), 154 (46).

Competitive Diazotizations (Table 3). A 0.22M aq. $NaNO_2$ soln. (200 µl) was cooled in an ice-bath and added dropwise within ½ h to an ice-cold and well stirred CF_3 COOH soln. (1.8 ml) containing 2-amino-1*H*-imidazole sulfate (5.28 mg, 0.02 mmol) as well as an equivalent concentration of lysine or arginine derivatives, if necessary *(Table 3)*. After 30 min of additional stirring on ice, the mixture was analyzed by UV. If necessary (*Table 3, Entries 4* and *9*), another 200 µl of $NaNO_2$ soln. was added (total, 2.2 equiv.) and the reaction carried out and analyzed as previously.

Enantiomeric Purity of **5** *and* **6**. The coupling reaction between **5** and FDAA (L) was achieved according to [14]: 200 µl of 18 mM *Marfey*'s reagent *(Pierce)* in acetone are added to 100 µl of 4 mM aq. amino-acid soln. and 40 µl of 1M aq. $NaHCO_3$. The mixture (pH 8) was stirred for 1 h at 40° and then treated with 20 µl of 2 N HCl. The obtained mixture was diluted 10 times with CH_3CN/H_2O 1:1, and 30-µl samples were taken for HPLC (*Varian Vista 5500,* column *Supersphere 100 RP-18e,* 125 × 4 mm *Merck,* temp. 40°, detection 340 nm; *A:* 95% 0.05 M $(Et_3N)_3PO_4$ buffer pH 3, 5% CH_3CN *LiChrosolv Merck; B:* CH_3CN *LiChrosolv Merck;* gradient 10% *B* to 50% *B* within 45 min with a flow rate of 1 ml/min): D,L-diastereoisomer at 34.85 min, L,L-diastereoisomer at 35.55 min, ratio 5:95.

An identical procedure was used to determine the enantiomeric purity of **6**, after a previous treatment of **6** with CF_3COOH at 0°: L,L-diastereoisomer at 24.38 min, D,L-diastereoisomer at 26.38 min, ratio 96.5:3.5.

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