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REGIOSELECTIVE ACYLATION OF 3,8-DIAMINO-5-ETHYL-6-PHENYL-PHENANTRIDIUM BROMIDE. PREPARATION OF POTENTIAL NEW TRYPANOCIDES

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ABSTRACT

A methodology was developed allowing regioselective modification of the trypanocide 3,8-diamino-5-ethyl-6-phenyl phenantridium bromide (ethidium bromide, homidium). Direct acylation of ethidium bromide preferentially yielded the C-8 amino substituted derivative. However, a reaction sequence including the reduction of the phenantridium ring, N-acylation and reoxidation allowed diacylation of the aminogroups in C-3 and C-8 position. A series of C-8 and C-3,8 acylated, respectively diacylated derivatives were prepared, aiming to improve the therapeutical index of the parent drug.

INTRODUCTION

3,8-Diamino-5-ethyl-6-phenyl-phenantridium bromide (ethidium bromide or homidium) (fig.1) is currently used as trypanocidal drug. Literature data are largely restricted to the study of the pharmacokinetics and pharmacodynamics, and to the use of this compound in elucidating the conformation of DNA and RNA strings.

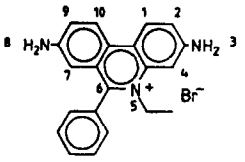


Fig. 1 : Ethidium bromide

The scarce synthesis efforts were made to develop either new derivatives suitable for the study of DNA and RNA, or new water soluble pigments (1-4). The early papers by Walls (5, 6) and by Watkins (7) are, to our knowledge, the only reports describing the synthesis of chemical analogues or derivatives of ethidium bromide with the intention of obtaining compounds with improved pharmaceutical properties.

In this paper the regioselectivity observed during the acylation of ethidium bromide with amino acids is reported. This regioselectivity is due to the considerable desactivation of the C-3 aniline group by resonance with the 5-ammonium function. Moreover, face to face dimerisation of ethidium bromide (8,9) (fig.2) has to be taken into account. These two phenomena determine to a large extent the whole chemistry of ethidium bromide.

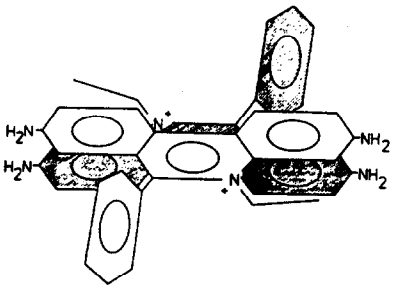
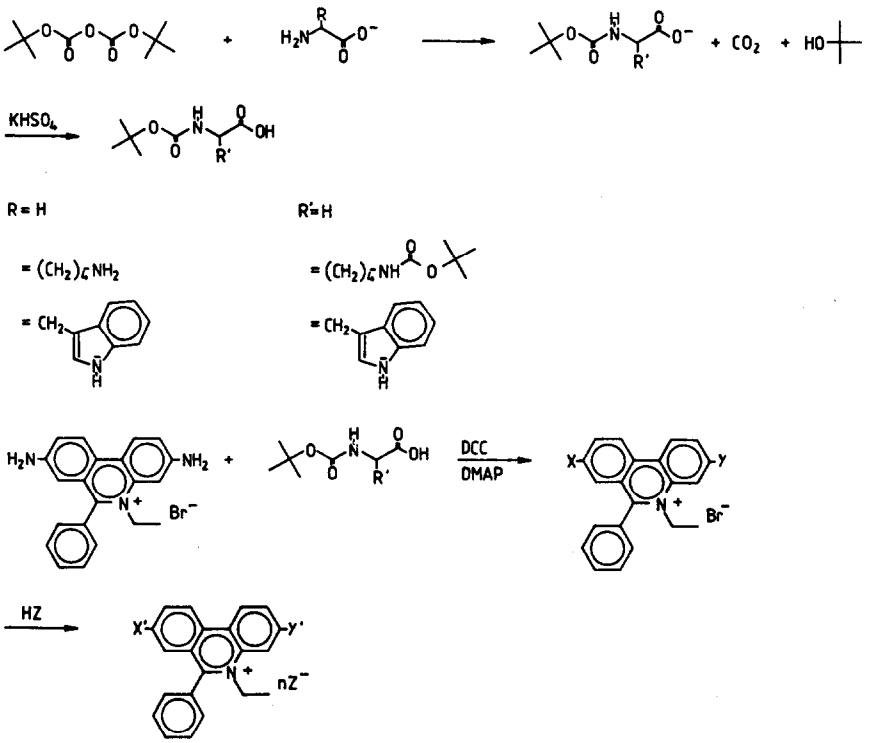


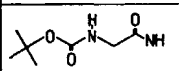
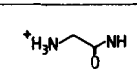
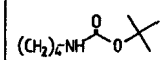
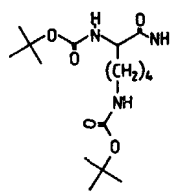
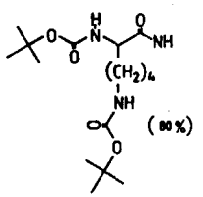
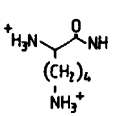
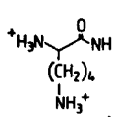
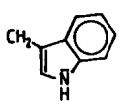
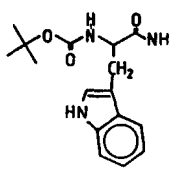
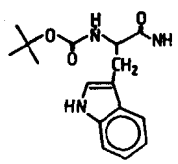
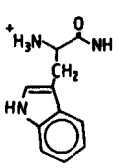
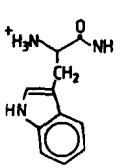
Fig.2 : Face to face dimer formed by ethidium bromide in solution.

RESULTS AND DISCUSSION

Direct acylation of ethidium bromide with protected amino acids occured as shown in scheme 1. The tertiary butyloxycarbonyl group (BOC) was selected as amino protecting group.

Scheme 1



R'	X	Y	HZ	X'	Y'	nZ^-
H		NH_2	CF_3COOH		NH_3^+	$3CF_3COO^-$
		 (80 %) NH_2 (20 %)	HOAc/HCl		 (80 %) NH_3^+ (20 %)	$5Cl^-$ $4Cl^-$
			CF_3COOH			$3CF_3COO^-$

Results obtained for BOC-glycine, diBOC-lysine and BOC-tryptophane

The regioselectivity of the acylation is investigated by proton NMR spectroscopy after acidolysis of the BOC group. Starting from an interpretation of the NMR spectrum of ethidium bromide, based on literature data (4,8), the spectra of the acylated derivatives were analysed. The chemical shift of the aromatic protons governs the essential information on the regioselectivity. The shifts differences for N-8-glycyl ethidium trifluoroacetate compared to the parent ethidium bromide itself are summarized in table 1.

Table 1 : Chemical shift differences for the aromatic protons of ethidium bromide and N-8-glycyl ethidium trifluoroacetate (in D_2O).

the aromatic protons	ethidium bromide (δ in ppm)	N-8-glycyl ethidium ($\Delta\delta$ in ppm)
H^1	8.10	+ 0.25
H^2	7.30	+ 0.02
H^4	7.26	+ 0.32
H^7	6.37	+ 1.05
H^9	7.20	+ 0.74
H^{10}	7.99	+ 0.38

Given the major chemical shift differences for the protons H^7, H^9 and H^{10} and the relatively minor differences for the protons H^1, H^2 and H^4, the acylation must have occured on the C-8 aniline function. HPLC analysis of N-8-(BOC-glycyl) ethidium bromide showed that below 1% of residual ethidium bromide and below 0.5% of the N-3,8-diacylated product was still present in the compound. Also elemental analysis proved the selectivity.

The analysis of the spectrum of N-3,8-ditryptophanyl ethidium trifluoroacetate is complicated by the indol protons. However, four protons of the phenantridium ring can easily be assigned. The chemical shift differences relative to ethidium bromide (in DMSO) are given in table 2.
The NMR spectrum of N-3,8-ditryptophanyl ethidium trifluoroacetate shows a remarkable anisotropy caused by the 6-phenyl group. The difference in chemical shift between both CH protons of the two tryptophanyl residues is around 0.2ppm. From the NMR data it can be concluded that both the C-3 and C-8 aniline functions are acylated.

Table 2 : Chemical shift differences for the aromatic protons of ethidium bromide and N-3,8-ditryptophanyl ethidium trifluoroacetate (in DMSO d_6).

the aromatic protons	ethidium bromide (δ in ppm)*	N-3,8-tryptophanyl (Δδ in ppm)
H^1	8.58	+ 0.72
H^2	7.48	+ 0.87
H^9	7.29	+ 1.11
H^{10}	8.52	+ 0.73

* spectrum : see ref. 8

The aromatic part of the spectrum of N-3,8-dilysyl ethidium chloride shows a complex pattern, which can be assigned to a mixture of a N-8 acylated and a N-3,8 acylated derivative. The ratio of both reaction products is 80/20 (N-3,8/N-8), as calculated from the NMR integrations.

The remarkable influence of the amino acid side chain on the regioselectivity of the reaction can be accounted to the desactivation of the C-3 aniline function by the ammonium function in position 5. This forms a kinetic restriction for acylation of the C-3 aniline group. Further more, it can be anticipated that the dimerisation phenomenon described for ethidium bromide can also occur with N-acylated derivatives, resulting in a shielding of the C-3-aniline group (fig. 3).

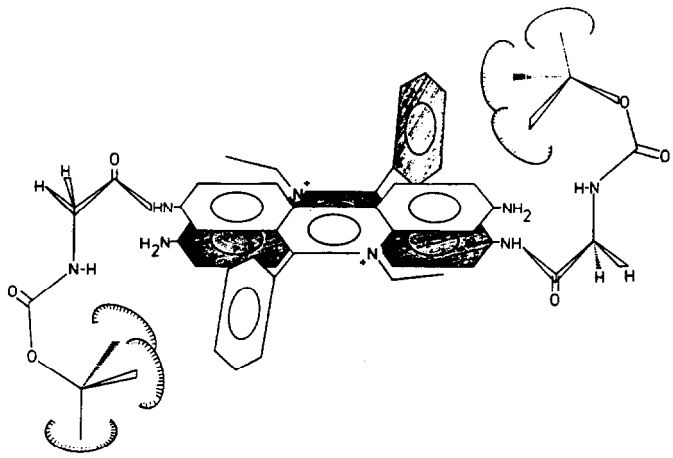


Fig. 3 : Shielding of the C-3 aniline function by face to face dimerisation.

Under the given reaction conditions, the concentration of free N-8-(BOC-glycyl) ethidium bromide is apparently so low that the reaction virtually

stops at that stage. Intermolecular interactions are responsible for the regioselectivity of the acylation reaction of ethidium bromide with BOC-glycine.

It can be anticipated that increasing bulkiness of the amino acid side chain increases the sterical hindrance and hampers the dimer formation (fig. 4). As a result, the C-3 aniline function becomes more susceptible for reaction.

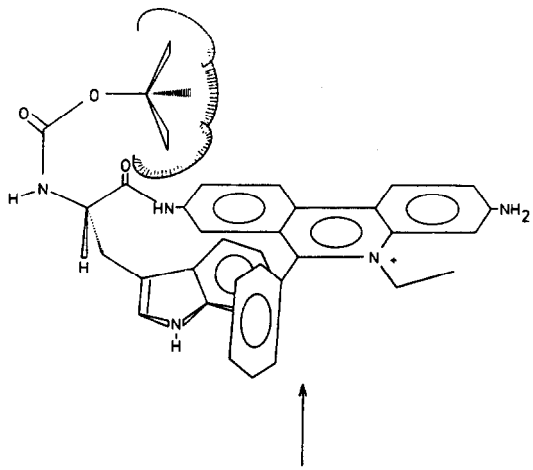


Fig. 4 : Sterical hindrance caused by bulky amino acid side chains.

This hypothesis is substantiated by the results obtained during coupling with di-BOC-lysine, having a bulky but flexible side chain, and with BOC tryptophan having a bulky but rigid side chain. The flexibility of the di-BOC-lysine side chain apparently allows dimerisation to some extent, while the rigidity of the tryptophan side chain causes much larger sterical restrictions for dimerisation. Hence, in the latter case the C-3 aniline function is fully accessible and the reaction goes to completion.

For the preparation of the N-3,8-diglycyl derivative the C-3 aniline function has to be made fully accessible. It was demonstrated by Thomas and Roques (8) that sodium borohydride reduced ethidium bromide no longer showed intercallation into the DNA double helix.

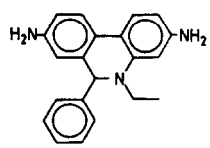
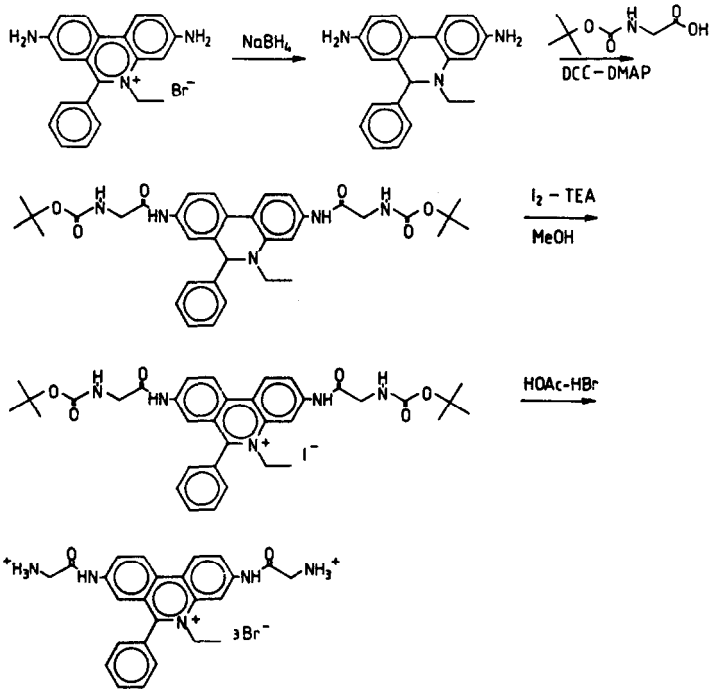


Fig. 5 : Sodium borohydride reduced ethidium bromide.

It was assumed that reduced ethidium bromide has lost its ability to dimerize. Given the reoxidation of reduced ethidium bromide is feasible, the latter must provide us with a handy approach to the preparation of ethidium bromide derivatives acylated on both the C-3 and C-8 aniline functions. For the reduction, the method described by Elderfield and Wark for the reduction of quinolium salts was applied (10).
The reoxidation to N-3,8-di(BOC glycyl) ethidium iodide is shown in scheme 2.

Scheme 2



The intermediates were not isolated because of their light and air sensitivity The chemical shifts of the aromatic protons are given in table 3.

The NMR spectrum of N-3,8-di(BOC glycyl) ethidium iodide shows a remarkable long range phenyl anisotropy (δ for the BOC groups : 0.05 ppm). The considerable downfield shift of all aromatic protons indicates that acylation has occured on both the C-3 and C-8 aniline function. The NMR data further prove that reoxidation was carried out with success, which was further confirmed by elemental analysis.
These data substantiate our hypothesis that reduced ethidium bromide has lost its ability to dimerize and provides the possibility for reaction on both amino functions.

Table 3 : Chemical shift of the aromatic protons of N-3,8-di(BOC glycyl) ethidium iodide compared to ethidium bromide (DMSO d_6).

the aromatic protons	ethidium bromide (δ in ppm)	N-3,8-di(BOC glycyl) ($\Delta\delta$ in ppm)
H^1	8.58	+ 0.57
H^2	7.48	+ 0.77
H^4	7.32	+ 1.78
H^7	6.18	+ 1.77
H^9	7.29	+ 1.16
H^{10}	8.52	+ 0.53

CONCLUSION

In this work, the feasibility for selective acylation of the C-3 and C-8 aniline functions of ethidium bromide has been demonstrated. The regioselectivity was attributed to intermolecular interactions, namely face to face dimerization of ethidium bromide derivatives. Sterical hindrance by bulky amino acid side chains proved to hamper the formation of the dimers sufficiently as to achieve quantitative acylation on both the C-3 and C-8 amino function.

By reduction of the phenantridium ring, ethidium bromide loses its ability to dimerize, which makes both aniline functions available for reaction. The reoxidation of the reduced intermediates was carried out with success. The biological evaluation of the ethidium derivatives will be discussed in a forthcoming publication.

EXPERIMENTAL PART

Materials :

NMR spectra were recorded on a 200 MHz (WP-Bruker) and a 360 MHz (WP-Bruker) NMR spectrometer. TLC analyses were run on Merck Kieselgel 60 F_{254} plates. The HPLC analysis was effected on a $^{10}\mu$ Boundapack Waters C-18 column, with UV detection.

NMR analysis of ethidium bromide :

360 MHz ^1H NMR spectrum of ethidium bromide in D_2O : the methyl protons of the 5-ethyl group : δ=1.3, m=3 (3H); the methylene protons of the 5-ethyl group : δ=4.43, m=4 (2H); H^1 : δ=8.10, m=2, J_{1-2}=8.8 Hz (1H); H^2 : δ=7.30, m=2x2, J_{2-1}=8.8 Hz, J_{2-4}=2 Hz (1H); H^4 : δ=7.26, J_{4-2}=2 Hz; H^7 : δ=6.37, m=2,

J_{7-9}=2 Hz (1H); H^9 : δ=7.20, m=2x2, J_{9-7}=2 Hz , J_{9-10}=8.8 Hz (1H); H^{10} : δ=7.99, m=2, J_{10-9}=8.8 Hz (1H); the ortho and para protons of the 6-phenyl group : δ=7.70-7.80, m (3H); the meta protons of the 6-phenyl group : δ=7.26-7.30, m (2H).

The preparation of N-8-glycyl ethidium trifluoroacetate :

To a solution of 250 mg (0.63 mmol) ethidium bromide in 15 ml dry DMF, 270 mg (126 mmol) BOC-glycine, 260 mg (1.26mmol) DCC and 60 mg (0.49 mmol) DMAP are added. The reaction is allowed to continue for 24 hours under nitrogen at 0°C and protected from light. The solvent is removed under reduced pressure. 50 ml methanol/ether (1/3) is added to the oily residue. After 48 hours storage in the refrigerator, an orange cristalline product is separated from the solution. The product is isolated by filtration, washed with ether and dried under vacuum (yield: 70%).

TLC analysis (eluent MeOH/NH_3 (0.5%)) : a bright orange spot R_f=0.3.

200 MHz ^1H NMR data (DMSO d_6) : the methyl protons of the BOC groups : δ=1.38, m=1; the methyl protons of the 5-ethyl group : δ=1.44, m=3 (together 12 H); the methylene protons of the BOC-glycine side chain : δ=3.81, m=2 (2H); the methylene protons of the 5-ethyl group : δ=4.62, m=4 (2H); the C-3 aniline protons : δ=6.77, m=1 (2H); the urethane proton : δ=7.10, m=3 (1H); H^1 : δ=8.86, m=2 (1H); H^2 : δ=7.52, m=2 (1H); H^4 + the 6-phenyl group : δ=7.86, m (6H); H^7 : δ= 7.57, m=1 (1H); H^9 : δ=8.38, m=2 (1H); H^{10} : δ=8.95, m=2 (1H).

Elemental analysis : $C_{28}H_{31}O_3N_4Br$		C	H	N
(551.48)	expected	60.98%	5.67%	10.15%
	found	61.05%	5.83%	9.98%

HPLC analysis (eluent : acetonitrile/water (80/20) 0.03% $HClO_4$, column : 10_μ Boundapack Waters C-18 (30 x 0.5 cm, UV detection at 290nm) : elution time 4 min. 25 sec.

Impurities : < 1% ethidium bromide (elution time : 4 min. 50 sec.) and <0.5% N-3,8-di(BOC-glycyl) ethidium bromide (elution time : 3 min. 12 sec.)

200 mg (0.36 mmol) of N-8-(BOC-glycyl) ethidium bromide is dissolved in 5 ml trifluoroacetic acid. The mixture is stirred for 3 hours. The reaction product is precipitated in 200 ml ether, which yields a cristalline residue. The mixture was allowed to stand over night. The residue is isolated by filtration, washed with ether and dried under vacuum (over all yield 60%).

360 MHz ^1H NMR data (D_2O) : H^1 : δ=8.35, m=2, J_{1-2}=8.9 Hz (1H); H^2 : δ=7.32, m=2, J_{2-1}=8.9 Hz (1H); H^4 : δ=7.58, m=1 (1H); H^7 : δ=7.42, m=1 (1H); H^9 : δ=7.94, m=2, J_{9-10}=9 Hz (1H); H^{10} : δ=8.37, m=2, J_{10-9}=9Hz (1H); the para and meta protons of the 6-phenyl group : δ=7.8-7.9, m (3H); the ortho protons of

the 6-phenyl group : δ=7.52, m=2 (2H); the CH_2 of N-8-glycyl substituent : δ=4, m=1; the CH_2 of the N-5-ethyl group δ=4.63, m=4, $J_{CH2-CH3}$=7.3 Hz (2H); the CH_3 of the N-5-ethyl group : δ=1.46, m=3, $J_{CH2-CH3}$=7.3 Hz (3H).

The preparation of N-3,8-dilysyl ethidium chloride :

To a solution of 50 mg (0.13 mmol) ethidium bromide in 2 ml dry DMF a large excess of diBOC lysine, DCC and a catalytic amount of DMAP are added. The reaction is carried out under nitrogen atmosphere, protected from light, at 0°C. The reaction was allowed to continue for 36 hours. TLC analysis showed two spots, one intense yellow spot R_f=0.8 and a minor orange spot R_f=0.75 (eluent : methanol/ 0.5% NH_3). The BOC groups were hydrolysed without isolating the intermediate products, by adding 5 ml of acetic acid satured with HCl. The reaction was allowed to continue for 2 hours. The product was precipitated in ether. After filtration and washing with ether, the product was dried in vacuum. The isolated product was analysed by NMR spectroscopy. The aromatic system of the ^1H NMR spectrum showed the presence of two products namely N-3,8-dilysyl ethidium bromide and N-8-lysyl ethidium bromide (yield : 70%).

360 MHz ^1H NMR data : the CH_3 of the N-5-ethyl group : δ=1.58, m=3; the - and δ-CH_2 of the lysine side chain : three complex multiplets between δ=1.68 and δ=1.9; the β-CH_2 of the lysine side chain : complex multiplet between δ=1.9 and δ=2.3; the -CH_2 of the lysine side chain : δ=3 and δ=3.05, m=3; the α-CH of the lysine side chain : δ=4.15, m and δ=4.35, m ; the CH_2 of the N-5-ethyl group : δ=4.75, m=4; the aromatic system H^2 (N-8) : δ=7.58, m=4; the 6-phenyl group (N-3,8 and N-8) : δ=7.79, complex multiplet; H^7 (N-8) : δ=8.08, m=2; H^7 (N-3,8) : δ=8.25, m=2; H^9 (N-8) and H^2 (N-3,8) : δ=8.43, m=2x4; H^9 (N-3,8) : δ=8.55, m=4; H^1 and H^{10} (N-8) : δ=8.88, m=2x2; H^{10} (N-3,8) : δ=9.08, m=2; H^1 (N-3,8) : δ=9.13, m=2; H^4 (N-3,8) : 9.18, m=2 (solvent MeOH d_3).

(N-8) = N-8-lysyl ethidium bromide (20% based on NMR integration)

(N-3,8) = N-3,8-dilysyl ethidium bromide (80% based on NMR integration).

The preparation of N-3,8-ditryptophanyl ethidium trifluoroacetate :

440 mg (1.45 mmol) BOC-tryptophane, 298 mg (1.45 mmol) DCC and 50 mg (0.41 mmol) DMAP is added to a solution of 100 mg (0.25 mmol) ethidium bromide in dry DMF. The reaction is allowed to continue for 20 hours at 0°C, protected from light and under inert atmosphere. The BOC groups are hydrolyzed without isolating the intermediate BOC protected reaction products, by adding 6 ml of trifluoro acetic acid. After 2 hours stirring the product is precipitated in ether yielding a yellow cristalline precipitate. The product is isolated by filtration, washed several times with ether and dried under vacuum (yield :

75%).
200 MHz ^1H NMR data (DMSO d_6) : the CH_3 of the N-5-ethyl group : δ=1.6, m=3 (3H); the CH of the N-8-tryptophanyl substituent : δ=4.3, m=3 (1H); the CH of the N-3-tryptophanyl substituent : δ=4.5, m=3 (1H); the CH_2 of the N-5-ethyl group : δ=4.7, m=4 (2H); H^1: δ=9.30, m=2; H^2 : δ=8.35, m=2; H^4 : δ=9.05, m=1; H^7 : δ=8.15, m=1; H^9 : δ=8.40, m=2; H^{10} : δ=9.25, m=2; the 6-phenyl group : δ=7.9, m; the indol protons : $H^{2'}$: δ=7.30 and δ=7.40, m=1; $H^{4'}$: δ=7.88 and δ=7.98, m=2; $H^{5'}$: δ=7, m=3; $H^{6'}$: δ=7.15, m=3; $H^{7'}$: δ=7.42, m=2.

The preparation of N-3,8-diglycyl ethidium bromide :
The preparation of reduced ethidium bromide :
200 mg (0.50 mmol) of ethidium bromide is dissolved in 6 ml absolute methanol. 88 mg $NaBH_4$ is added at 0°C. The reaction is carried out under nitrogen, in absence of light and at 0°C for 24 hours. The solvent is evaporated under reduced pressure and 6 ml of a 3% NaOH solution is added. The solution is extracted 6 times with 20 ml ether. The ether fractions are pooled and dried over anhydrous Na_2SO_4. Ether is evaporated under reduced pressure and a light brown cristalline residue is obtained which is recristallized from ether. For spectroscopical and conformational data see reference 8.

The coupling of BOC glycine to reduced ethidium :
To a solution of 250 mg (0.79 mmol) of reduced ethidium in 4 ml dry acetonitrile, a 5 fold excess of BOC-glycine and DCC is added to obtain a 100% acylation of the amino functions. The reaction must be carried out in the dark and under nitrogen due to the instability of reduced phenantridium molecules. The reaction is allowed to continue for 20 hours. After 20 hours the DCU formed is removed by filtration and the acetonitrile is removed in vacuum. Separation at this stage is not necessary.

Reoxidation of N-3,8-di(BOC glycyl)amino-6-ethyl-5-phenyl-5,6-dihydrophenantridine :
The crude reduced product is dissolved in 20 ml methanol. 0.3 ml triethylamine and a slight excess of I_2 (206mg, 0.81mmol)are added. The mixture is refluxed until the solution becomes orange. The solution is concentrated to 5 ml and the product is precipitated in ether. Redissolving in methanol, followed by precipitation in ether is used to purify the product (yield: 80%).
200 MHz ^1H NMR data (DMSO d_6) : the BOC groups : δ=1.4, m=1, δ=1.45, m=1; the CH_3 of the N-5-ethyl group: δ=1.55, m=3 (together 21H); the CH_2 of the N-8-BOC-glycyl group : δ=3.7, m=2 (2H); the CH_2 of the N-3-BOC-glycyl group : δ=3.9, m=2 (2H); the CH_2 of the N-5-ethyl group : δ=4.7, m=2 (2H); the urethane proton of the N-8-BOC-glycyl group : δ=7.05, m=3 (1H); the urethane

proton of the N-3-BOC-glycyl group : δ=7.2, m=3 (1H); H^1 : δ=9.15, m=2 (1H); H^2 : δ=8.25, m=2 (1H); H^4 : δ=9.1, m=1 (1H); H^7 : δ=7.95, m=1 (1H); H^9 : δ=8.45, m=2 (1H); H^{10} : δ=9.05, m=2 (1H); the 6-phenyl group : δ=7.85, m (5H)

elemental analysis : $C_{35}H_{42}O_6N_5I$		C	H	N
(755.65)	expected	55.63%	5.60%	9.27%
	found	55.84%	5.70%	9.15%

TLC analysis (eluent MeOH/NH_3 0.5%) : a bright yellow spot R_f=0.7

The hydrolysis of the BOC groups :

N-3,8-di(BOC glycyl)ethidium iodide is dissolved in 5 ml acetic acid satured with HBr. After 2 hours the product is precipitated in ether, which resulted in a yellow cristalline product. (overall yield : 60%)

200 MHz ^{1}H NMR data (DMSO d_6) : the CH_3 of the N-5-ethyl group : δ=1.51, m=3, $J_{CH2-CH3}$=7 Hz (3H); the CH_2 of the N-8-glycyl group : δ=3.8, m=1 (2H); the CH_2 of the N-3-glycyl group : δ=4.0, m=1 (2H); the CH_2 of the N-5-ethyl group : δ=4.65, m=4 (2H); H^1 : δ=9.21, m=2, J_{H1-H2}=10 Hz (1H); H^2 : δ=8.28, m=4, J_{H2-H1}=10 Hz, J_{H2-H4}=2 Hz (1H); H^4 : δ=9.03, m=2, J_{H4-H2}=2 Hz (1H); H^7 : δ=8.11, m=2, J_{H7-H9}=2.5 Hz (1H); H^9 : δ=8.35, m=4, J_{H9-H10}=9.2 Hz, J_{H9-H7}= 2.5Hz (1H); H^{10} : δ=9.13, m=2, J_{H10-H9}=9.2Hz (1H); the 6-phenyl group : δ=7.82, m (5H)

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