BASE-PROMOTED CLEAVAGE OF α-N-NBD*-PEPTIDES

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1. Introduction

We describe in this communication a possible new approach to sequential peptide degradation which is based on the fact that 7-alkylamino-4-nitrobenz-2-oxa-1,3-diazoles (NBD-Amines), equation 1, are reasonably strong acids with pK_a values of about 10. If the conjugate base of such an acid, a nitrogen with a negative charge, is at the N-terminal end of a peptide, it is six atoms removed from the carbonyl carbon atom of the second peptide bond, with which it can react. The result of this reaction is the ultimate removal of two amino acids, from the original peptide.

2. Materials and methods

Buffers were prepared with reagents of analytic grade from British Drug Houses.

Gly-gly-L-phe was a product of the Sigma Chemical Company, St. Louis, Mo U.S.A. The phenylalanine sample used was from a collection of chromatographically pure amino acids from British Drug Houses. Pentaglycine, triglycine, diglycine and diketopiperazine were from Fluka AG, Zug, Switzerland.

NBD-Cl was synthesized as described by Boulton et al. [1].

The synthesis of α-*N*-NBD-peptides is illustrated with the example of pentaglycine. 31 mg of pentaglycine (0.1 mmol) was neutralised with 2 ml of 0.1 N NaOH and added to 100 mg of NBD-Cl in 200 ml of ethanol. The reaction time at room temp. was 16 hr.

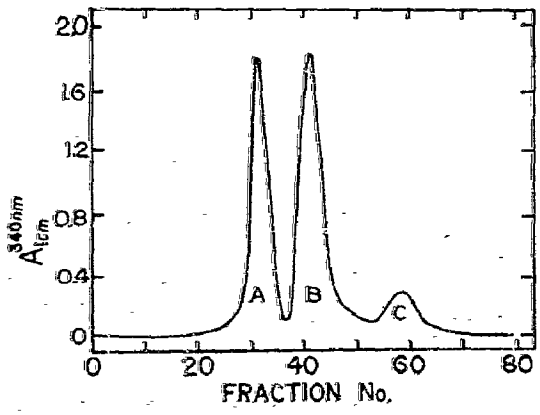


Fig. 1. Synthesis of NBD-$(Gly)_5$. Gel filtration of reaction mixture. Column 84 X 1.5 cm Sephadex G 25 fine. Eluant: distilled water; Fraction size 4 ml; Flow rate 10 ml/hr. A: NBD-$(Gly)_5$; B: NBD-Cl; C: NBD-OH.

* Non-standard abbreviations: NBD: 4-nitrobenz-2-oxa-1,3-diazolyl; BBD: 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole; NBD-Cl: 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NBD-OH: 7-hydroxy-4-nitrobenz-2-oxa-1,3-diazole; $(Gly)_3$: Triglycine and $(Gly)_5$: Pentaglycine.

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Table 1
Paper chromatographic behavior of NBD-peptides.

NBD-peptide	R_f	
	Solvent 1	Solvent 2
NBD-Gly–Gly–L-Phe	0.022	0.91
NBD-$(Gly)_5$	0.043	0.47
NBD-$(Gly)_3$	0.051	0.67

Solvent 1: Ethyl acetate, pyridine, water 8:2:1; Solvent 2: n-butanol, acetic acid, water 40:6:15.

Table 2
pK_a values for representative NBD-monoalkylamines.

Compound	pK_a
BBD	9.8
NBD-L-Val	10.0
NBD-L-Arg	10.0
NBD-L-Arg	9.9
NBD-L-Trp	9.9
NBD-L-Ser	10.0

The reaction mixture was concentrated and separated by gel filtration on a column of Sephadex G-25, fig. 1. Peak A representing α-*N*-NBD-$(Gly)_5$ was lyophilized and further purified by high voltage electrophoresis on Whatman No. 3 paper at 3000 V, pH 4.7 for 90 min. The desired product was eluted from the paper with distilled water and lyophilized with a yield of 10 mg. The product was homogeneous on paper chromatography with two different solvent systems (table 1) as well as on high voltage electrophoresis on paper at pH 1.9. It was devoid of any ninhydrin-reactive material, illustrating complete reaction between NBD-Cl and $(Gly)_5$.

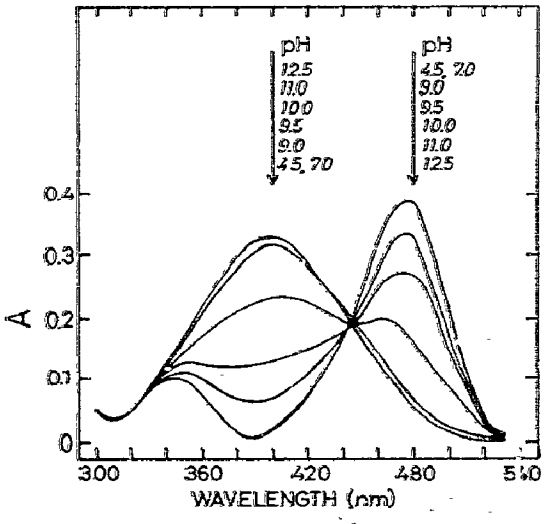
Absorbance measurements were carried out with a Pye Unicam spectrophotometer 1800.

3. Results

3.1. *Effects of pH on the ultraviolet-visible spect of NBD-amines*

Fig. 2a shows the changes in the ultraviolet-visible spectrum of BBD [2], as a function of pH. The two peaks at 340 nm and 480 nm disappear with increasing pH and a new one at 400 nm appears with ϵ_{400} = 20 700 and an isosbestic point at 442 nm (ϵ_{442} = 13 000). This change is reversible. A plot of the change in absorbance either at 400 nm or at 480 nm as a function of pH, fig. 2b, is a typical titration curve with a midpoint at pH 9.8.

Similar results have been obtained for other NBD-alkylamines as shown in table 2.



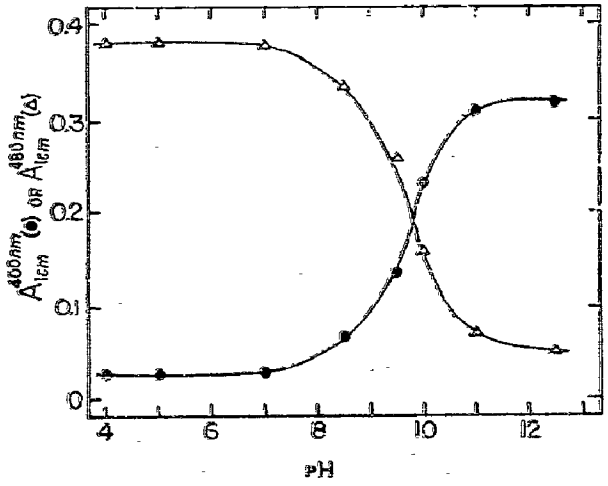
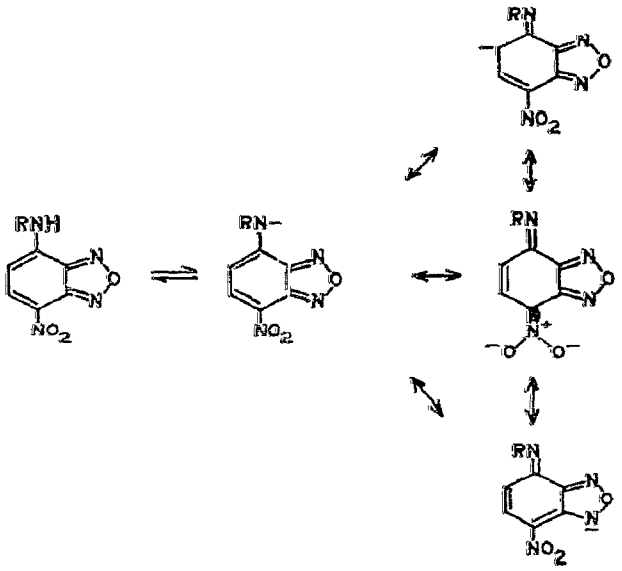


Fig. 2. a) Spectrophotometric titration of 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole (BBD). Concentration 1.55×10^{-5} M. b) Changes in absorbance of BBD at 400 nm (•) and 480 nm (○) as a function of pH. Buffers were 0.1 M in concentration. pH 4.5, acetate; pH 7, 11, 12.5, phosphate; pH 9, 9.5 and 10, borate.

EQUATION I



Scheme 1.

As opposed to this, N-NBD-morpholine [3] with a tertiary nitrogen does not give such a result. Instead the compound is quantitatively hydrolyzed to yield the phenol, NBD-OH, and morpholine at pH values above 9. E.g. at pH 10.3 the half-life for the hydrolysis is 20.5 min.

These observations have been interpreted in terms of the ionization of the secondary amine in NBD-amines to give a conjugate base with a negative charge on the nitrogen. This conjugate base is resonance-stabilized as shown in equation 1.

This puts NBD-amines in the same class of nitrogen acids such as phthalimide (pK_a = 8.30) [4].

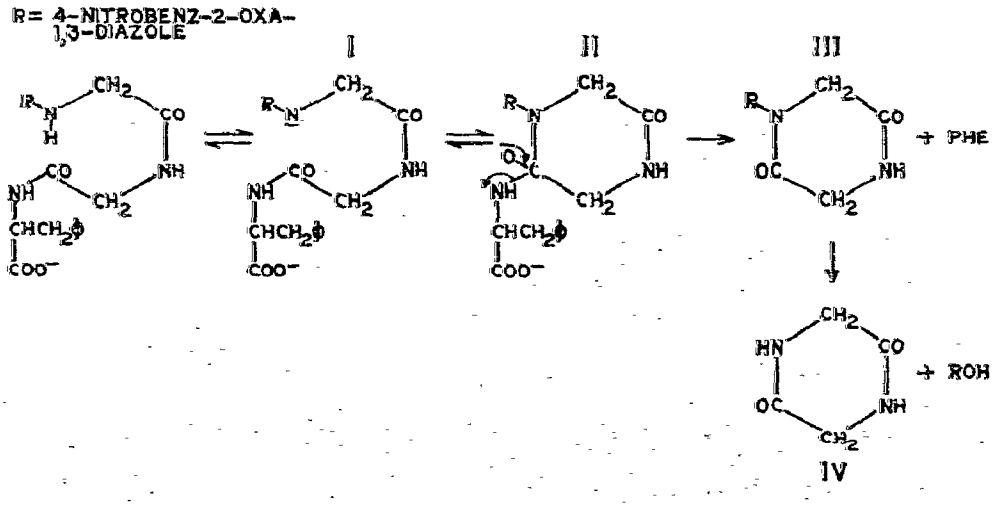
3.2. *Peptide bond cleavage*

A nitrogen anion such as that in fig. 1 is a reactive species in spite of the delocalization of the electron as shown. If it is generated in α-*N*-NBD-peptides, it is sterically well positioned to react with the carbonyl carbon atom of the second peptide bond, being six atoms removed from this site. The result of such a reaction should be a cleavage of the second peptide bond.

We have tested this hypothesis by studying the stability of the simple α-*N*-NBD-peptides in table 1 in aqueous buffers at pH values close to the expected pK_a (table 2).

The NBD-peptides were dissolved in 0.05 M borate buffer pH 10.3. The solutions were at 50°C for 6 hr

EQUATION II



Scheme 2.

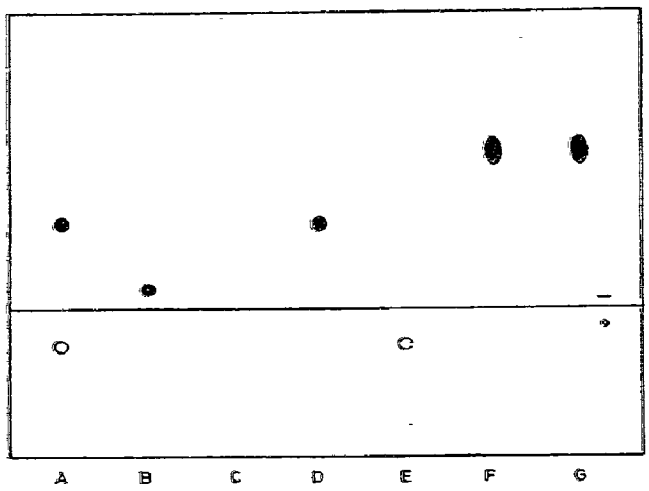


Fig. 3. Electrophoretogram of α-*N*-NBD–Gly–Gly–Phe hydrolysate. Electrophoresis was carried out at pH 1.9, 3000 V (33 V/cm) for 75 min of A: NBD-Gly–Gly–Phe at pH 10.3 (6 hr); B: NBD-Gly–Gly–Phe at pH 7.0 (6 hr); C: Diketopiperazine at pH 10.3 (6 hr); D: Phe E, NBD-OH; F: Gly–Gly–Phe at pH 10.3 (6 hr); G: Gly–Gly–Phe in distilled water (6 hr). NBD-peptide and NBD-OH are easily visible because of their characteristic color. Other compounds were detected by spraying paper with 0.2% ninhydrin.

after which they were acidified to pH 4 with concentrated formic acid and lyophilized. In control experiments the NBD-peptides were dissolved in 0.05 M phosphate buffer pH 7.0, and the original unmodified peptides in the borate buffer at pH 10.3.

Fig. 3 is a diagram of the electrophoretogram at pH 1.9 for the experiments with NBD-Gly–Gly–Phe (0.1 mM). The following results are evident: a) There is a complete disappearance of NBD-Gly–Gly–Phe at pH 10.3; b) This disappearance is accompanied by the formation of the phenol, NBD-OH, and phenylalanine with no other ninhydrin positive material formed; c) Incubation of NBD-Gly–Gly–Phe at pH 7.0 at 50°C for 6 hr leaves the modified peptide unchanged; d) At pH 10.3 and 50°C no peptide bond cleavage is observable using Gly–Gly–Phe, or diketopiperazine.

Similar results have been obtained with NBD-$(Gly)_3$ which yielded glycine and NBD-OH, and NBD-$(Gly)_5$, which yielded triglycerine and NBD-OH.

4. Discussion

The following reaction scheme is the simplest that can be proposed to account for the results described above. (See equation II.)

The attack of the nitrogen anion in I at the carbonyl carbon of the second peptide bond produces the cyclic intermediate II which can either return to I or loose phenylalanine to yield the NBD-diketopiperazine III. In an analogous manner to the instability of NBD-morpholine, III will hydrolyse under the conditions of the reaction to produce the diketopiperazine IV and NBD-OH. This step is probably the major driving force for the reaction. Even though diketopiperazine cannot be directly demonstrated, the scheme is consistent with the release of phenol.

While the conditions for NBD-peptide formation and degradation used in this series of experiments are by no means optimal, the results suggest a new approach to the sequential chemical degradation of peptides characterised by the removal of two amino acid residues at each round of degradation. A major feature of it lies in the fact that both the coupling reaction, with NBD-Cl, and degradation step can be carried out in aqueous media under much milder conditions than those employed for the standard Edman degradation [5]. As such it could be employed with native proteins for the removal of N-terminal amino acids in structure function studies.

There are however two important limitations inherent in the method. First, when proline is at the N-terminal end of a peptide, the NBD derivative, like NBD-morpholine, will be unstable under the conditions for degradation. Such peptides will therefore be resistant to the degradative procedure. The same problem exists for an N-terminal glutaminyl residue in which the possibility of pyroglutamic acid formation under the degradative conditions cannot be ruled out.

Secondly, since the steric requirement for reaction are stringent, the effect of the greater degrees of conformational freedom in larger peptides may lead to different cleavage patterns from that observed with the shorter peptides used in this work.

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