

Partial Structure of Papiliochrome, the Yellow Wing Pigment of the Papilionid Butterflies

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Dedicated to Professor Adolf Butenandt on the Occasion of His 75th Birthday

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Papiliochrome II, N-(β -alanyl)-L-noradrenaline, Catecholamine, NMR, GC-MS

Papiliochrome II, one of the yellow wing pigments of the Papilionid butterfly, *Papilio xuthus*, can be split into L-kynurenine and a catecholamine derivative, SN-1, by 10^{-3} N HCl. The latter is hydrolyzed by N HCl into β -alanine and noradrenaline. The structure of SN-1 has been elucidated as N-(β -alanyl)-L-noradrenaline by NMR and MS data as well as by synthesis. Preparation of the N-hydroxysuccinimide ester of benzyloxycarbonyl- β -alanine, its condensation with L-noradrenaline to the corresponding benzyloxycarbonyl protected SN-1 and of N-(β -alanyl)-L-noradrenaline by hydrogenolysis of the protecting group are described.

The yellow wing pigment of the swallowtail butterfly, *Papilio xuthus*, is composed of two groups of compounds. The main pigment, Papiliochrome II, has been investigated since 1954 by Umebachi [1] and some of the physical and chemical properties have already been reported [2]. Remarkably enough, it neither belongs to the class of pterins nor to the ommochromes, both of which are common insect pigments. Papiliochrome II is very labile and is readily decomposed to L-kynurenine and the DOPamine derivative SN-1 by weak mineral acid already. By hydrolysis in 1 N HCl at 100 °C for two hours [3, 4], SN-1 is further broken down into β -alanine and the DOPamine derivative SN-1a. SN-1 has been presumed to be a N-(β -alanyl) DOPamine derivative. These results are supported by ^{14}C -incorporation of the injected precursors [^{14}C]tryptophan, [^{14}C]DOPamine, and [^{14}C] β -alanine into the yellow wing pigments [2, 3, 5].

Umebachi suggested that Papiliochrome II might be a molecular complex of kynurenine and SN-1. Our first step for structural elucidation was therefore to analyse this DOPamine derivative. The present paper reports the identification of the DOPamine moiety as noradrenaline, the elucidation of SN-1 as N-(β -alanyl)-noradrenaline, and the synthesis of this peptide.

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Material and Methods

All chemicals used were of analytical grade. SN-1 was prepared from wings of male adults of *P. xuthus* as described already [4].

Derivatives of SN-1 were prepared in the following way: About 0.1 mg of SN-1 was sealed in a small glass ampoule together with 5–10 μl N,O-bis-(trimethylsilyl)-acetamide (BSA) or the deuterated compound under nitrogen and heated at 200 °C for 5 min. For mass spectrometric analysis a direct inlet system with a temperature program between 20–300 °C was used. The TMS derivative evaporated at 220 °C. For preparation of the TFA derivative, about 0.1 mg of SN-1 was sealed with 5–10 μl trifluoroacetic acid anhydride and a trace of 3-(dimethylamino-)pyridine under nitrogen and kept at 150 °C for 5 min. This derivative too was directly introduced into the mass spectrometer; evaporation temperature 220 °C. For hydrolysis, about 1 mg of SN-1 in 1 ml 3 N HCl was kept under nitrogen in a sealed ampoule for 1 hour at 100 °C. The acid was then evaporated under reduced pressure, 100 μl BSA were added, and the mixture was heated under nitrogen for 10 min at 200 °C. The reaction mixture was analysed in a capillary GC-MS-system.

A CH7 A-MAT mass spectrometer coupled with a glass capillary column SE-30 (LKB, 25 m length, 0.35 mm i.d., He flux 0.5 cm^3 per min), and connected with a computer SS-100 MAT, was used. The glass capillary was heated in a linear program from 100–325 °C with a temperature rate of 10 °C/min. Spectra were measured with 70 EV, emission 1 mA, ion source temperature 250 °C.



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NMR spectra were measured with a Bruker WH90 Fourier instrument, solvent $D_2O/MeOH-D_4/DCI$, TMS as internal standard.

N-Hydroxysuccinimide ester of benzyloxycarbonyl- β -alanine (I)

Benzyloxycarbonyl- β -alanine (17.85 g, 80 mmol) and hydroxysuccinimide (9.20 g, 80 mmol) were dissolved in 300 ml of anhydrous tetrahydrofuran. The solution was cooled to 4 °C, an icecold solution of dicyclohexylcarbodiimide (18.15 g, 80 mmol + 10%) in anhydrous tetrahydrofuran was added, and the mixture was stirred at 4 °C for a period of 48 h. The dicyclohexyl-urea which had formed was removed by filtration and washed with tetrahydrofuran. The filtrate was evaporated to dryness to yield a yellow oil. Recrystallization from isopropanol yielded 23.45 g of white crystals (91%). Apart from the well-known decomposition of *N*-hydroxysuccinimide esters on silica-gel [6], the compound was pure according to TLC in ether, tetrahydrofuran and isopropanol. M.P. 83° – 84 °C. NMR (Acetone- $D_6/D_2O/DCI$): δ 2.92 (methylene groups of hydroxysuccinimide), 2.94 and 3.55 (methylene groups of β -alanine), 5.21 (methylene group of *Z*-residue), 7.37 (aromatic protons).

Anal. Calcd. for $C_{15}H_{16}N_2O_6$: C, 56.25; H, 5.03; N, 8.75. Found: C, 56.26; H, 4.95; N, 8.77.

N-(Benzyloxycarbonyl- β -alanyl)-L-noradrenaline (II)

L-Noradrenaline (1.69 g, 10 mmol) was suspended in 35 ml of dimethylformamide, and 1.1 ml (10 mmol) *N*-methylmorpholine together with a solution of benzyloxycarbonyl- β -alanine-hydroxysuccinimide ester (3.84 g, 12 mmol) in another 35 ml of dimethylformamide was added. In order to minimize autoxidation, the solvent used was previously flushed with hydrogen and the reaction vessel was closed under hydrogen. After stirring for 48 h in the cold, a clear solution had formed. 2-(1-Piperazinyl)-ethylamine [7] (0.52 ml, 0.4 equiv.) was added and the solution was allowed to adjust to room temperature for 1 h. The solvent was removed in a rotary evaporator under vacuum; ethyl acetate (100 ml) and 0.5 N H_2SO_4 (50 ml) were added. Following shaking and separation, the aqueous layer was extracted four times with 20 ml of ethyl acetate. The ethyl acetate phases were combined and the resulting solution was washed 3 times with

saturated sodium chloride solution. After drying over anhydrous sodium sulfate, evaporation of the ethyl acetate left 3.61 g of a colourless oil (96% yield) which soon crystallized. Recrystallization from 130 ml of ethyl acetate gave 3.07 g (82%) of a white product. A second crystallization from $MeOH-H_2O$ (1 : 9) yielded 2.58 g of white needles which were found to be pure according to TLC in *n*-butanol/ $HOAc/H_2O$ (6 : 2 : 2). M.P. 124 – 125 °C. NMR (Acetone- D_6/D_2O): δ 2.44 and 3.40 (methylene groups of β -alanine), 3.38 (methylene group of noradrenaline), 4.61 (methine group of noradrenaline), 5.07 (methylene group of *Z*-residue), 6.75 (aromatic protons of noradrenaline), 7.40 (aromatic protons of *Z*-residue).

Anal. Calcd. for $C_{19}H_{22}N_2O_6$: C, 60.95; H, 5.92; N, 7.48. Found: C, 60.96; H, 5.90; N, 7.48.

N- β -alanyl-L-noradrenaline (III)

Z- β -alanyl-L-noradrenaline (1.2 g, 3.2 mmol) was dissolved in 70 ml of $MeOH-H_2O$ (4 : 1), the solution was flushed with N_2 for 10 min and palladium black was added as a catalyst. The suspension was stirred magnetically, while a stream of hydrogen gas was passed into it at room temperature. After 24 h, no starting material could be detected by TLC in *n*-butanol/ $HOAc/H_2O$ (6 : 2 : 2). The catalyst was removed by filtration, 100 ml of water were added and the methanol was removed in a rotary evaporator. The remaining aqueous solution was lyophilized, yielding 0.75 g (97.5%) of III which was found to be homogeneous according to TLC in the above mentioned system. The substance is hygroscopic. M.P.: 134 – 137 °C. NMR (D_2O): δ 2.49 and 3.03 (methylene groups of β -alanine), 3.46 (methylene group of noradrenaline), 4.68 (methine group of noradrenaline), 6.80 (aromatic protons).

Anal. Calcd. for $C_{11}H_{16}N_2O_4$: C, 54.99; H, 6.71; N, 11.66. Found: C, 54.33; H, 6.69; N, 11.59.

Results and Discussion

Structure of SN-1

Papiliochrome II and its SN-1 component can be collected from wings in small amounts only. Structural elucidation of SN-1 as *N*-(β -alanyl)-noradrenaline was achieved by a combination of nuclear magnetic resonance and mass spectroscopic data.

The 3,4-dihydroxyphenyl (catechol) nucleus of the molecule is confirmed primarily through the aromatic protons (multiplet at 6.80 ppm) displayed

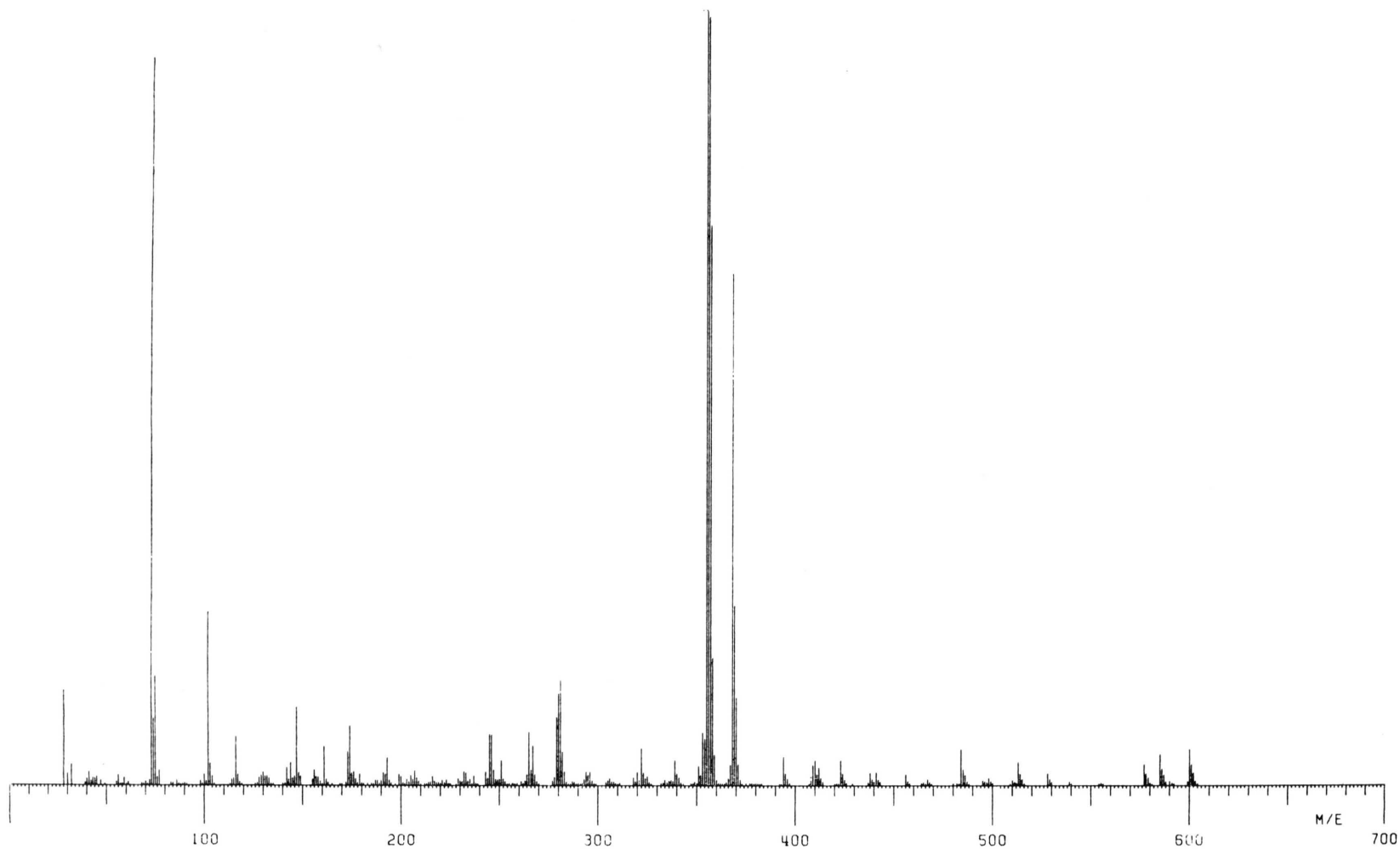


Fig. 1. Mass spectrum of penta-(trimethylsilyl)-SN-1. The reaction mixture was introduced through the direct inlet system.

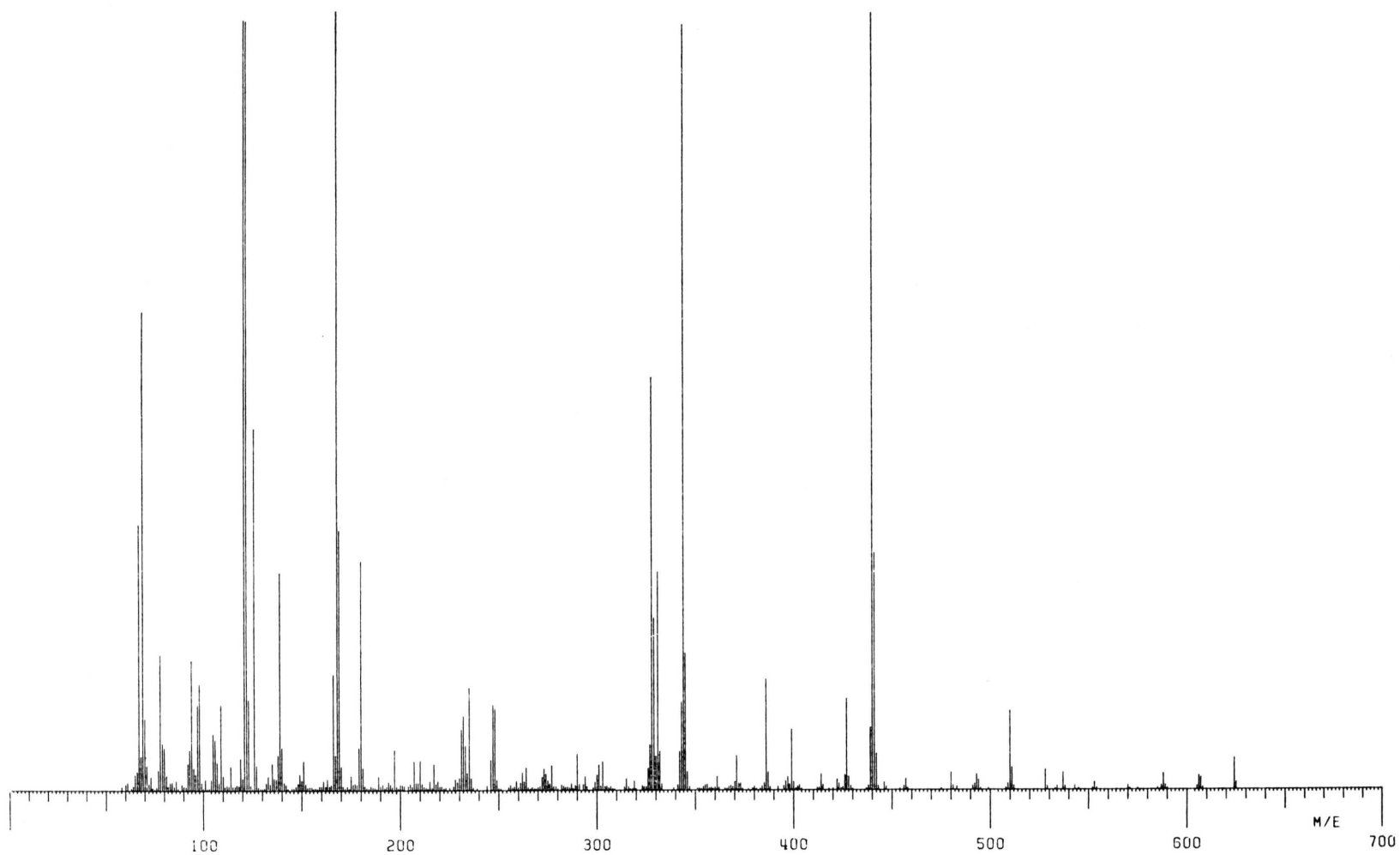


Fig. 2. Mass spectrum of tetra-(trifluoroacetyl)-SN-1. The reaction mixture was introduced through the direct inlet system.

in the nuclear magnetic resonance data of SN-1 (Table). The other upfield signals of the alkyl substituent can be identified in combination with the mass spectra of the derivatives penta-(trimethylsilyl)-SN-1 and tetra-(trifluoroacetyl)-SN-1.

The molecular ion peak of the TMS derivative (Fig. 1) is at m/e 600. By comparison with the deuterated D_9 -derivative, molecular weight 645, one counts five TMS reactive groups and a molecular weight of 240 for SN-1. The most prominent ion from the TMS derivative is at m/e 355, and from the D_9 -TMS derivative at m/e 382. These ions fit into the structure of a 1-hydroxymethylenecatechol tris(trimethylsilyl ether)cation and therefore point towards the β -hydroxyl group contained in noradrenaline. Such a structure is supported by the further mass fragmentation pattern, by the A_2B system of the protons at position 4 and 5 in the nuclear magnetic resonance spectrum (Table), and finally confirmed by comparison with the mass spectrum of synthetic TMS-noradrenaline. Combination of these facts proves identity of SN-1a with noradrenaline. The linkage of noradrenaline with β -alanine (A_2B_2 system of the protons at position 1 and 2, Table) is shown by the McLafferty fragment at m/e 368 for the TMS (Fig. 1), and at m/e 395 for the D_9 -TMS derivative of SN-1. The corre-

sponding fragment represents the ion at m/e 440 in the mass spectrum of tetra-(trifluoroacetyl)-SN-1 (Fig. 2). All further MS studies equally confirm the structure of SN-1 as N-(β -alanyl-)noradrenaline.

The products from hydrolysis of SN-1 in 1 N HCl, 100 °C, were separated as TMS derivatives in a capillary gas chromatograph coupled with the mass spectrometer. Only tris(trimethylsilyl) β -alanine (Fig. 3, peak I) and penta(trimethylsilyl)-noradrenaline (Fig. 3, peak II) could be detected. Minor products are bis(trimethylsilyl)- β -alanine (155 °C), tris and tetra-(trimethylsilyl)-noradrenaline (220–240 °C). Compared with the corresponding synthetic compounds, retention time in the column as well as mass spectra were identical.

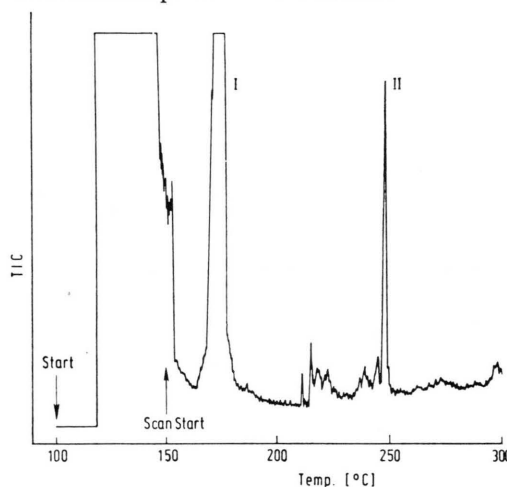


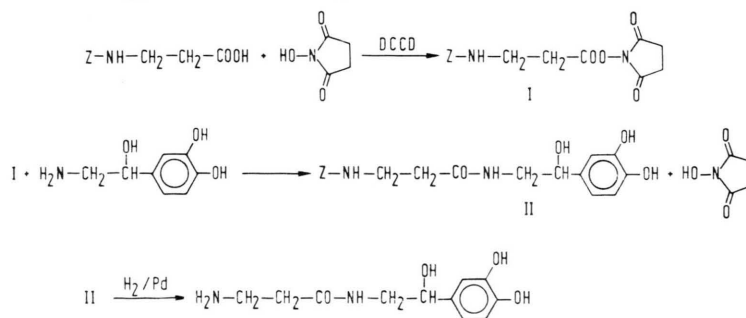
Fig. 3. Capillary GCMS-chromatogram of TMS-derivatives obtained after acid hydrolysis of SN-1. Peak I is identical with synthetic tri-TMS- β -alanine, peak II with penta-TMS-noradrenaline. Mass resolution about 800, mass range m/e 25–1,000, scan frequency 10 spectra per min, temperature program 10 °C per min.

Table. 1H Nuclear magnetic resonance data (D_2O /methanol- D_4/DCI ; TMS, $\delta=0.0$ ppm) of SN-1.

$H_2N-CH_2-CH_2-CO-NH-CH_2-\overset{\overset{OH}{ }}{CH}-\text{C}_6H_3(OH)_2$					
1	2	3	4	5	6
Position	Spin system		ppm		
1	triplet		3.22		
2	triplet		2.65		
4	doublet		3.45		
5	triplet		4.68		
6	multiplet		6.80		

Synthesis of N-(β -alanyl)-L-noradrenaline, SN-1

The synthesis of this compound which has some structural similarity with a peptide, was performed in the following sequence:



For the acylation of noradrenaline by the amino acid β -alanine, the general principles of peptide synthesis, namely protection of the amino group and activation of the carboxyl function, had to be applied. The well known tendency of catecholamines towards autoxidation suggested the use of the benzyloxycarbonyl-group (Z) to protect the amino-group of β -alanine, as this residue may be removed by hydrogenation. Problems might arise from a possible nucleophilic attack on the three hydroxyl groups in the noradrenaline molecule. In combination, however, with suitable activation methods, amino acids with non-protected hydroxyl functions in the side chain have previously been used in peptide synthesis: Inouye *et al.* [8] describe the coupling of Z- β -alanine with tyrosine methyl ester with unmasked oxygen function; a series of peptides containing 3,4-dihydroxyphenylalanine (DOPA) was successfully prepared without protection of the phenolic hydroxyl groups (Losse *et al.* [9], O'Neill *et al.* [10]). N-Hydroxysuccinimide esters were introduced into peptide synthesis by Anderson *et al.* [11, 12]. Their high reactivity with amino-groups as compared to a rather modest sensitivity against hydrolysis and alcoholysis [13] prompted us to choose this method of activation for the desired acylation of the primary amino group of L-noradrenaline in the presence of two phenolic and one aliphatic non-protected hydroxyl groups. The N-hydroxysuccinimide ester of benzyloxycarbonyl- β -

alanine has not been described in literature: it was synthesized according to the method of Anderson *et al.* [11, 12] and characterized as described in Material and Methods.

Biochemical aspects

Natural occurrence and chemical synthesis of N-(β -alanyl-)noradrenaline have not been described previously. Its occurrence in the wings of the Papilionidae and its combination with L-kynurenine in a ratio of 1 : 1 to form papiliochrome is remarkable for the butterflies. Kynurenine is derived from tryptophan metabolism and it is precursor for the class of ommochromes [14]. Catecholamines are derived from phenylalanine and tyrosine. N-Acetyldopamine was found in the blowfly, *Calliphora erythrocephala* [15], and is responsible there for the tanning of the cuticle. In other flies, β -alanine seems to be the tanning agent and to be responsible for compaction of the cuticle in the fruit fly, *Drosophila melanogaster* [16]. It is possible for this reason that deposition of Papiliochrome in the wings during development of the adult butterfly serves to control pool sizes of the aromatic amino acids tryptophan and phenylalanine as well as that of β -alanine. As noradrenaline is also present in the nervous system of insects, another possibility is that N-(β -alanyl)-noradrenaline is in some way involved in the hormone function of catecholamines.

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