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DES–Nα^1–ACETYL–α–MELANOTROPIN

A Synthetic Substrate for Specific N-Terminal Directed Enzymatic Acetylation

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Application of the 2-methylsulfonylethyloxycarbonyl group for temporary amino protection enables the synthesis from one precursor of des-Nα^1-acetyl-α-MSH, the two mono N-acetylated forms (in positions I and II) and the diacetyl form of this tridecapeptide amide. The free tridecapeptide amide, although structurally unrelated to the normal substrate, was recognized by an enzyme occurring in calf eye-lens tissue. The product of the enzymatic reaction was exclusively α-MSH. Partial sequences derived from the N-terminus were less rapidly acetylated or not at all, depending on their chain length. The enzyme, therefore, appears to direct its activity to free N-terminal α-amino groups of peptides exceeding a certain critical chain length. Acetylation of ε-amino functions did not occur.

N-terminal acetylation of proteins is a widespread phenomenon in prokariotic and eukariotic cell (Strous *et al.*, 1973, Pestana & Pitot, 1975). About one-third of the weight of the vertebrate eye lens consists of proteins, mainly the so-called crystallins, 80% of which are N-terminally acetylated. It could be demonstrated previously that acetylation occurs while the growing peptide chain is still on the ribosome (Strous *et al.*, 1974) Triggering of the acetylation reaction requires a protruding chain of approximately 20 amino acyl residues. Since the translation products of the α-crystallin messengers are acetylated in heterologous systems (Berns *et al.*, 1972, *a,b*) it appears sensible to refer to the acetylating mechanism as non-exclusive for the parent cell species. The study of this enzymic process has been seriously hampered by the lack of a suitable substrate. Therefore, a synthetic peptide with a free N-terminus, which is known to occur in nature solely* in its N-terminal acetylated form, was supposed to constitute a proper model for specific N-terminal acetylation, by this enzyme. Des-Nα^1-acetyl-α-melanotropin (I), which contains free amino groups in the positions 1 and 11, is such a model. (Designation and abbreviation according to IUPAC-IUB Commission on Biochemical Nomenclature.) Only a specific N-terminal acetylation of this tridecapeptide amide would produce naturally occurring α-MSH.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-

Arg-Trp-Gly-Lys-Pro-Val-NH_2 (I)

This substrate, therefore, enables the detection of an enzyme, which acetylates one of these functions, preferentially by discrimination between N^{α} and N^{ϵ}.

* The recent detection of des-N^{α}-acetyl-α-melanotropin in camel pituitaries, is the first exception to this rule (Li *et al.*, 1975).

MATERIALS AND METHODS

The substrate and three reference compounds (III–V, Scheme 1 and Table 1) were obtained from one intermediate (II). This compound was prepared from partially protected

$$\begin{array}{c} \text{OBu}^t \\ | \\ \text{Boc-Ser-Tyr-Ser-Met-Glu-His-} \end{array}$$

$$\begin{array}{c} \text{Msc} \\ | \\ \text{Phe-Arg-Trp-Gly-Lys-Pro-Val-NH}_2 \end{array} \quad \text{(II)}$$

β-corticotrophine-(1-10)-decapeptide (Schwyzer & Kappeler, 1961) and the new tripeptide derivative H-Lys-(Msc)Pro-Val-NH_2. HCl (the symbol Msc denotes the novel 2-methylsulfonylethyloxycarbonyl group (Tesser & Balvert-Geers, 1975; Van Nispen, 1974) Conversion of II to I was performed by treatment with a strong base during a very short period (15–30 sec) and subsequent removal of the *t*-butyl functions by acidolysis. Conversion of II to the respective mono-acetylated forms was performed by insertion of an acetylation reaction between the two deprotecting reactions, which can also be performed in an inversed sequence (cf. Scheme 1). The acetylation was carried out with *p*-nitrophenyl acetate. The di-acetylated compound (IV) was obtained from either III or V. Chromatographic solvents for t.l.c. on silica (precoated plates containing a fluoresence indicator) were the following: (A) BuOH-AcOH-w = 10:1:3, (B) BuOH-AcOH-w = 4:1:1, (C) Propanol-2–25%NH_3-w = 80:1:19, (D) Propanol-2-AcOH-w = 77:4:19, (E) BuOH-Pyr-AcOH-w = 38:24:8:30, (F) Propanol-2–25%NH_3-w = 3:1:1.

Preparation of H-Lys(Msc)-Pro-Val-NH_2.HCl

Boc-Lys(Msc)-ONp was prepared from Boc-Lys (Msc)-OH (Tesser & Balvert-Geers, 1975) using the general method of Bodansky & du Vigneaud (1959). (Yield: 87%, m.p. 104–106°, $[\alpha]_D^{20} = -20.9°$ (c = 1, EtOAc.) *Analysis* calc. for $C_{21}H_{31}N_3O_{10}S$: (517.55) C, 48.73; H, 6.04; N, 8.12; S, 6.20. *Found:* C, 48.50; H, 6.0; N, 8.0; S, 6.0.)

Boc-Lys(Msc)-Pro-Val-NH_2. A slight molar excess of H-Pro-Val-NH_2 (Schwyzer *et al.*, 1963) was added to a 0.2 M solution of Boc-Lys (Msc)-ONp in dimethyl formamide and the reaction mixture was left for 16 h at room temperature. The solvent was removed *in vacuo* and replaced by ether, whereupon the product solidified. The amorphous solid was washed with ether, dissolved in ethyl acetate and extracted with potassium hydrogen sulfate solution (0.5M) saturated with sodium chloride. (Yield: 88%, m.p. 76°, $[\alpha]_D^{20} = -59.0°$ (c = 1.1 in MeOH) *Analysis* calc. for $C_{25}H_{45}N_5O_9S$ (591.2): C, 50.74; H, 7.67; N, 11.84; S. 5.42. *Found:* C, 50.50; H, 7.70; N, 11.65; S, 5.25.

H-Lys(Msc)-Pro-Val-NH_2.HCl. Deprotection of the α-amino group in the protected tripeptide amide was carried out with a tenfold excess of dry hydrochloric acid in ethyl acetate. The precipitated mono hydrochloride was dissolved in hot isopropyl alcohol; it precipitated in an amorphous state. (Yield: 80%, m.p. 100° (unsharp), $[\alpha]_D^{20} = -40.2°$ (c = 1.2 in methanol). *Analysis* calc. for $C_{20}H_{37}N_5O_7S$. HCl.½H_2O (537.10): C, 44.71; H, 7.32; N, 13.04; S, 5.97; Cl, 6.60. Found: C, 44.85; H, 7.50; N, 12.75; S, 6.10; Cl, 6.55.)

Preparation of II. To a solution of 1.537 g (1 mmol) of [Boc-Ser[1], Glu[5](OBu^t)]-β-corticotrophin-(1-10)-decapeptide (Schwyzer & Kappeler, 1961) and 1.056 g (2 mmol) of H-Lys(Msc)-Pro-Val-NH_2.HCl in 50 ml of pure dimethylformamide, 0.270 g (2 mmol) of N-hydroxybenzotriazole (König & Geiger, 1970) were added. Following the addition of 100 mg (1 mmol) of N-methyl-morpholine 0.309 g (1.5 mmol) of solid dicyclohexylcarbodiimide were dissolved in the solution. After 16 h at 45° under nitrogen, the reaction mixture was cooled at 0° for some hours and then filtered. The filtrate was added dropwise to 200 ml of dry peroxide-free ether which was stirred vigorously. T.l.c. revealed that the decapeptide derivative had reacted completely. The crude II was purified by countercurrent distribution over 87 transfers using the system butanol-ethyl acetate-acetic acid-water (1:3:1:5).

Selective deprotection and acetylation

a) *Removal of t-butyl functions* was performed with 75% aqueous trifluoroacetic acid accord-

ing to the procedure of Riniker & Rittel (1970).

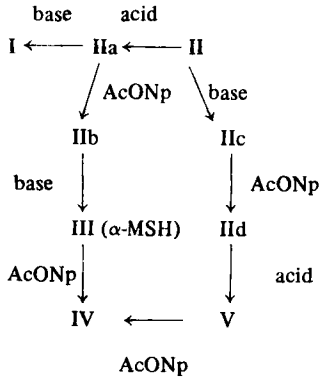
b) *Removal of the Msc function.* An alkaline reagent was prepared by mixing peroxide-free dioxan, methanol and aqueous NaOH (11.3 N) in the proportions 30:9:1 (base concentration 0.282 N). To a solution of about 100 mg (50 μmol) of the appropriate peptide derivative in 1.75 ml of dimethylformamide enough of the base was added to account for all ionizable protons, the proton liberated by the Msc-group, and to leave two equivalents in excess (concentration of the base in the mixture 0.1 N). After 20 sec the base was quenched with acetic acid (1 M in MeOH) and the product was precipitated with ether as indicated before. The precipitate was freed from sodium acetate by extraction of the product into aqueous butanol. The extracts were evaporated and the residue was lyophilized from 0.1 N acetic acid.

c) *Introduction of acetyl groups.* The partially protected intermediates were acetylated in dimethylformamide solution using two equivalents of *p*-nitrophenyl acetate. The acetylated compounds were isolated by precipitation with ether and lyophilization from 0.1 N acetic acid.

Enzymatic acetylations. The isolation and partial characterization of the acetylating enzyme from calf eye-lens tissue, as well as the conditions for the catalyzed reaction, have been

SCHEME 1

Syntheses of compounds I and III-V from II



Sequence of the operations as described in the section Materials and Methods under 'selective deprotection and acetylation'. The coding of the intermediates is explained in Table 1.

TABLE 1

Relevant characteristics of compounds I–V

Compound	R^1	R^2	R^3	Yield	$[\alpha]_D^{22}$ *	Bc*	A**	B	C	D	E	F
I	H	OH	H	57	−27.0	0.25					0.39	0.63
II	Boc	OBu^t	Msc	84	−29.0	0.50	0.16	0.31	0.51	0.55	0.62	0.70
IIa	H	OH	Msc	81	−27.8	0.50		0.08		0.16	0.42	0.68
IIb	Ac	OH	Msc	92	−30.8	0.50	0.09	0.18	0.33	0.56		
III (α-MSH)	Ac	OH	H	88	−48.8	0.41		0.10		0.14	0.45	0.60
	Ac	OH	H		−56.0	0.35						
IV	Ac	OH	Ac	82	−26.4	0.57		0.27		0.52		0.71
IIc	Boc	OBu^t	H	77	−34.0	0.50	0.10	0.22		0.34	0.58	
IId	Boc	OBu^t	Ac	88	−30.6	0.41		0.32		0.57	0.60	0.68
V	H	OH	Ac	90	−28.3	0.51		0.12		0.20	0.49	0.69

R_2 R_3

R^1-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2: Coding, specific rotations, yields and chromatographic behavior.

* Specific rotations were measured after equilibration of the compounds with the humidity of air. The solvent was AcOH-H_2O (9:1); the second value for α-MSH (compound III) was determined under conditions as stated by Schwyzer *et al.* (1963); c = 0.35 in AcOH-w = 1:9. Concentrations in g/100 ml solution.

**The solvent systems A-F are specified in the section Materials and Methods.

TABLE 2

Amino acid composition including ammonia of the ultimate compounds II and IV

Compound	Ser	Glu	Pro	Gly	Met	Val	Phe	Tyr	NH_3	Trp	Lys	His	Arg
II	1.89	1.00	1.02	1.01	0.96	0.98	0.97	0.97	1.16	0.36	1.01	1.00	1.01
IV	1.82	1.01	0.97	1.02	0.98	0.97	0.99	0.99	1.36	0.46	1.02	1.00	1.00
Expd.	2	1	1	1	1	1	1	1	1	1	1	1	1

Amino acid analyses were carried out on 24-h acid hydrolysates in 6N HCl "suprapur" of quality Merck (Darmstadt, Germany) without application of additives. The Tyr/Trp ratio was determined independently (Benzce & Schmid, 1957), and was found to be 0.96 in II and 0.98 in IV.

described elsewhere (Granger *et al.*, to be published).

RESULTS

Syntheses

Compound II, following countercurrent distribution was obtained as a homogeneous compound, giving the Pauly, Sakaguchi and Ehrlich reactions. It constituted the starting material for the reaction sequence of Scheme 1, which gives the order of successsion of the reactions described in the preceding section under *a-c*. The coding and yields of the resulting products, as well as their relevant characteristics, are given in Table 1. Samples of the first and the last products of the sequence (II and IV) were subjected to amino acid analysis (Spackman *et al.*, 1958) and determination of the Tyr/Trp ratio (Benzce & Schmid, 1957). The results are given in Table 2.

Enzymatic acetylation

Compound I was recognized by an enzyme system occurring in a cell-free extract of calf eye-lenses, and producing α-MSH by selective acetylation of the N-terminal seryl residue. Compound III (α-MSH) was not a substrate for the enzyme, indicating an absolute specificity for N-terminal acetylation. The enzyme required a chain length of more than four amino acyl residues, since the N-terminal tetrapeptide H-Ser-Tyr-Ser-Met-OH was not recognized as a substrate. However, the octapeptide resulting from a tryptic hydrolysis of I was acetylated enzymatically, but the reaction proceeded much faster with the complete tridecapeptide amide. The presence of a free lysyl residue in position 11 is not a prerequisite for the reaction to occur, since both IIa and V are recognized by the enzyme preparation, but the process runs about twice as fast if this function is free (Granger *et al.*, to be published).

DISCUSSION

The development of the Msc-protective function (Tesser & Balvert-Geers, 1975) for temporary amino protection has already offered a number of solutions to synthetic problems in peptide chemistry. Apart from its excellent acid stability, which meets the requirements set by Hirschman & Veber (1973), the group has the distinct advantage of improving the solubility of peptide derivatives in polar solvents as compared with the more familiar benzyloxycarbonyl and t-butyloxycarbonyl derivatives. By its resistance to hydrogenation, the Msc group combines well with the N-protective functions derived from benzyl alcohol, which in turn, similar to those of t-butanol, are stable to alkaline reagents. Removal of an Msc-group is performed by inducing β-elimination and therefore requires the presence of a strong base. The reaction is extremely rapid, provided the base concentration is made sufficiently high. At 0.1 molar concentrations, the deprotection is virtually complete within 5 sec. The amino function is ejected as the N-carboxylate, which acts as a protection for a secondary Michael-type alkylation of the deprotected group by the liberated methylsulfonyl ethylene (Tesser & Balvert-Geers, 1975). In the presence of methanol, the major fission product is 2-methylsulfonylethylmethyl ether. The Msc-group has been applied successfully in our laboratories during the syntheses of ACTH and α-MSH sequences (Van Nispen, 1974) and in several

other synthetic projects dealing with gastrin and cytochrome c. The applicability in the chemistry of insulin has been proven by Geiger *et al.* (1975 *a, b*). A detailed report on the chemistry of α-MSH, in which compound II was prepared and handled independently, has been the result of a cooperative investigation with Eberle *et al.* (1975).

The availability of the substrate described (compound I), enabled for the first time the demonstration of the existence of an acetylating enzyme with absolute specificity for N-terminal amino groups (Granger *et al.*, to be published). The reaction requires acetyl coenzyme-A as acetyl donor. Although two potential sites for N-acetylation are present in the substrate, the N-terminus is acylated exclusively. The enzyme requires a minimal chain length of eight amino acyl residues in the substrate to exert its activity. The fact that the occurrence of N-acetylated proteins having N-terminals other than those derived from serine, threonine, glycine, alanine and methionine is scarce, suggests that a certain specificity for recognition of the N-terminals in a substrate might exist.

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