NONINVASIVE CONTINUOUS MONITORING OF SOLID-PHASE PEPTIDE SYNTHESIS BY ACID-BASE INDICATOR*

Viktor KRCHŇÁK[a], Josef VÁGNER[a], Pavel ŠAFÁŘ[b] and Michal LEBL[b]

[a] *Léčiva-Pharmaceuticals, 143 10 Prague 4 and*
[b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*

Received December 23rd, 1987
Accepted February 6th, 1988

Dedicated to the memory of Dr Karel Blâha.

The course and the end point of the acylation of resin-bound amino groups in solid-phase peptide synthesis was monitored by a novel noninvasive qualitative and quantitative test based on the use of the acid-base indicator bromophenol blue.

Merrifield's solid-phase peptide synthesis[1,2] requires almost quantitative acylation of amino groups upon the peptidyl resin. Several methods of monitoring the course and completion of this reaction have been described (for a review see e.g.[2]), the ninhydrin test being the one most commonly used[3]. Recently, we have employed an indicative amount of 3,4-dihydro-3-hydroxy-4--oxo-1,2,3-benzotriazine to monitor the acylation of amino groups in our configuration of continuous-flow solid-phase peptide synthesis[4,5]. This triazine has been recommended by Atherton et al.[6] for the preparation of self-indicating activated esters. However, the colour change from yellow to white is not sufficiently conspicuous if one works on standard polystyrene resin that tends to be yellowish itself. Therefore, we attempted to find an alternative indicator that would be deprotonated by free amino groups on peptidyl resin and whose ionized form would be deeply coloured.

Among the several indicators tested, bromophenol blue (3',3",5',5"-tetrabromophenolsulfophtalein) displayed the best properties: the colour change from yellow (λ_{max} 429 nm) to dark blue (λ_{max} 600 nm) is remarkable, the sensitivity is high (molar absorption coefficient ε_{600} 91 800). The resin lacking free amino groups exposed to the solution of bromophenol blue becomes

* Part CCVI in the series Amino Acids and Peptides; Part CCV: Collect. Czech. Chem. Commun. *53*, 2473 (1988).

yellowish to yellowy-orange and when free amino groups are present the colour turns deep blue (not all acid-base indicators change their colour in the presence of free amino groups on resin). Bromocresol green and bromocresol purple displayed also satisfactory properties.

Acylation monitoring can be performed both continuously (a solution of bromophenol blue is simply added to the acylating agent or to the last washing solvent before coupling) or discontinuously (a sample of peptidyl resin is withdrawn from the reactor and treated with bromophenol blue); the free resin-bound amino groups are also easily determined quantitatively. The most important feature of the test, however, is the possibility to follow the course of the acylation continuously by sight. This advantage should be appreciated especially in multiple peptide synthesis where several couplings have to be followed and checked at the same time. The test is not destructive, the excess of bromophenol blue can be easily washed out by a solution of a base even if the indicator is present in an equimolar amount. We quantitatively followed the kinetics of one of the syntheses using picric acid[7], ninhydrin[8], and bromophenol blue (BB). The obtained results are summarized in Table I. The BB method gave comparable results, but it is simpler to perform and its further advantage over the ninhydrin test is that it can be repeated with the same sample several times.

TABLE I

Comparison of the various methods for the coupling step monitoring (coupling of Boc-Ala (3 eq.) onto the H-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-*p*-methylbenzhydrylamine-copoly(styrene-1% divinylbenzene)resin (1 g, 0.77 mmol/g) in dimethylformamide (15 ml) with dicyclohexylcarbodiimide (3 eq.), N-hydroxybenzotriazole (3 eq.), and 150 μl of 1% bromophenol blue solution in dimethylacetamide)

Time, min	Colour		Ninhydrin test[a]	$[NH_2]$,mmol/g		
	Beads	Supernatant		Picric acid[b]	Ninhydrin (quantitative[c])	BB[d]
5	blue	colourless	+++	0.303	0.286	0.292
10	blue	colourless	+++	0.161	0.153	0.156
20	blue-green	slightly yellow	+	0.042	0.035	0.049
40	yellow-green	yellow	±	0.013	0.015	0.012
80	yellow (trace of green)	yellow	–	0.004	0.003	0.003

[a] Ref.[3]; [b] ref.[7]; [c] ref.[8]; [d] this paper.

which can increase its reliability. Moreover, condensations involving aminoterminal proline can be monitored without difficulties.

The indicator does not get involved in any side reaction: we never observed unexpected products in crude peptides. In the blank acylation experiment (benzhydrylamine resin, bromophenol blue, dicyclohexylcarbodiimide, N-hydroxybenzotriazole in dimethylformamide in the absence of amino-protected amino acid) the starting deep blue colour of the resin did not change even after five weeks. After this period simple addition of Boc-Ala and fresh dicyclohexylcarbodiimide led to the coupling and therefore decoloration of the resin. To prove that the presence of the indicator did not deteriorate the quality of the synthesized peptide we have performed the comparative synthesis of two oxytocin analogs which were synthesized in our laboratory several times and whose properties are very well known (for the review see[9]) — deamino-oxytocin[10] and deamino-1-carba-oxytocin[11]. Both these peptides were carefully checked by chromatography in the stage of the crude reaction mixture (after cleavage from the resin and oxidative cyclization in the case of deamino-oxytocin) and no significant differences in the mixture composition were found. Both peptides were fully characterized in the pure form analytically (amino acid and elemental analyses), spectroscopically (^{1}H NMR spectrum at 500 MHz, CD spectrum) and pharmacologically (uterotonic and galactogogic activity). None of these tests revealed any significant differences. Yields of the pure compounds were comparable with those of the same peptides obtained in our laboratory previously.

The sensitivity of the monitoring is high; routinely bromophenol blue was used in only 1/1 000 molar equivalent of the amino groups. Nevertheless, even a full equivalent of bromophenol blue can be present in the reaction mixture without any apparent influence on the coupling (but not on the sensitivity of the test — see below).

The sensitivity of this monitoring method brings about its own limitations:
(i) The quality of the protected amino acids must be high, especially as far as the content of unprotected species is concerned. This high purity is in some cases very hard to achieve, for example in Boc-Gln or Boc-Asn.
(ii) Dimethylformamide used for the synthesis must be dimethylamine-free and no base can be added during the coupling step.
(iii) The nature of the base used in the neutralization step is very important if the classical Merrifield's resin is used. The residual chloromethyl groups form quaternary ammonium salts with the tertiary amine and this leads to blue colouring of the resin after the addition of the bromophenol blue. This colour is not washed by the tertiary base and quaternary ammonium salt must be used for its washing. (On the other hand, bromophenol blue makes possible quantitative determination of these strongly basic quaternary ammonium

groups on the resin.) Table II shows a comparison of triethylamine (TEA) and diisopropylamine (DIEA) in formation of these strongly basic groups on the resin. It is evident that triethylamine is not compatible with this monitoring method since the significant background colouring must be expected when synthesis is performed on chloromethylated polystyrene. On the other hand, bulky diisopropylethylamine does not cause this side reaction in significant extent. A one week exposure to diisopropylethylamine leads to twenty times lower quaternary ammonium ion formation than a short (30 min) exposure to triethylamine solution.

(iv) Only very low amount of monitoring dye can be used since it has the ability to decolourize itself by its leuko-form. Therefore the amount of the indicator applied to the resin should be lower than the quantity of the free amino group which we want to monitor. For example, if we want to observe the coupling, which is 99.9% complete, performed on 1 g of resin with the substitution 1 mmol/g, we must not apply more than 1 μmol of the bromophenol blue (i.e. 25 μl of 0.04 M solution). Therefore the analytical rule of the as low as possible amount of indicator must be followed.

To demonstrate the reliability of this monitoring method we applied it in the standard batchwise solid phase synthesis of several analogues of oxytocin ([2-D-tyrosine]deamino-1-carba-oxytocin, [2-O-methyltyrosine, 7-(3,4-dehydroproline)]deamino-1-carba-oxytocin, antiparallel dimer of deamino-1-carba--oxytocin, [2-phenylalanine]oxytocin, [2-D-phenylalanine]oxytocin, [2-alanine]-tocinoic acid, [2-alanine, 3-alanine]tocinoic acid, [2-alanine, 3-alanine, 4-alanine]tocinoic acid, [2-alanine, 3-alanine, 4-alanine, 5-alanine]tocinoic acid), somatostatin, tyrosyl-somatostatin, [5-norleucine]enkephalin. In all cases, we have obtained the usual composition of the crude reaction mixture and the pure peptides were obtained by the standard purification schedule (gel filtration on

TABLE II

Content of quaternary ammonium groups formed by exposure of chloromethylated polystyrene (0.81 mmol/g content of chlorine) to tertiary amines

Time, h	Quaternary ammonium groups, mmol/g	
	TEA	DIEA
0.5	0.006	–[a]
5	0.026	–[a]
48	0.124	1×10^{-4}
180	0.261	2.5×10^{-4}

[a] Not measurable.

Bio-Gel P-4 followed by preparative reversed-phase high performance liquid chromatography (HPLC)).

Beyond comparison is the applicability of BB method in the concurrent synthesis of peptides. This was demonstrated in the case of a simultaneous synthesis of six 20-residue peptides according to the multiple continuous-flow

TABLE III
Amino acid sequences of prepared peptide amides

Peptide	Virus Protein	Amino acid sequence
I	HIV-1 p24	Val-His-Ala-Gly-Pro-Ile-Ala-Pro-Gly-Gln--Met-Arg-Glu-Pro-Arg-Gly-Ser-Asp-Ile-Ala
II	HIV-2 p24	Pro-Ile-Pro-Gly-Pro-Leu-Pro-Ala-Gly-Gln--Leu-Arg-Glu-Pro-Arg-Gly-Ser-Asp-Ile-Ala
III	HIV-1 p31	Ile-Gln-Asn-Phe-Arg-Val-Tyr-Tyr-Arg-Asp--Ser-Arg-Asn-Pro-Leu-Trp-Lys-Gly-Pro-Ala
IV	HIV-2 p31	Leu-Lys-Asp-Phe-Arg-Val-Tyr-Phe-Arg-Glu--Gly-Arg-Asp-Gln-Leu-Trp-Lys-Gly-Pro-Gly
V	HIV-1 gp41	Arg-Pro-Glu-Gly-Ile-Glu-Glu-Glu-Gly-Gly--Glu-Arg-Asp-Arg-Asp-Arg-Ser-Ile-Arg-Leu
VI	HIV-2 gp41	Ala-Asn-Glu-Glu-Thr-Glu-Glu-Asp-Gly-Gly--Ser-Asn-Gly-Gly-Asp-Arg-Tyr-Trp-Pro-Trp

TABLE IV
Analytical data on individual peptides

Peptide	Yield, mg		R_t[a] min	Purity[b] %
	resin	peptide		
I	720	232	9.5	76
II	700	184	19.5	83
III	930	347	22.6	75
IV	810	257	16.8[c]	74
V	1 020	340	8.2	72
VI	740	272	4.1	71

[a] Retention times in HPLC (column 25 × 0.46 cm, Separon SGX C18, mobile phase: 60% methanol in water containing 0.1% TFA, flow rate 90 ml/h); [b] the area of the peak of the product to the area of all peaks in the crude product measured at 220 nm; [c] 65% MeOH.

solid phase peptide synthesis configuration described earlier[5]. The peptides were derived from the env, pol, and gag protein products of human immunodeficiency viruses (HIV-1 ref.[12] and HIV-2 ref.[13]) and contained the predicted recognition sites for B-cells[14] (for amino acid sequence, see Table III). Crude peptides were obtained in good yield and their purity was greater than 70% according to the reversed-phase HPLC (see Table IV). Crude peptides were purified to homogeneity by preparative reversed-phase HPLC and purified peptides afforded expected amino acid analyses.

EXPERIMENTAL

Qualitative Continuous Monitoring in Solid-Phase Peptide Synthesis Performed Batchwise

The syntheses were carried out in an ordinary shaker reactor. After the addition of the protected amino acid, hydroxybenzotriazole and dicyclohexylcarbodiimide in dimethylformamide or dichloromethane, three drops of a 1% solution of bromophenol blue in dimethylacetamide were added. The suspension turned dark blue. After the suspension in the reaction vessel turned greenish-yellow, the next step of the synthesis was carried out. All experimental details regarding the synthetic protocol, cleavage conditions and purification procedures were the same as described previously[15]. In the case of deamino-oxytocin and deamino-1-carba-oxytocin, we compared the products with the pure peptides prepared by solid-phase method without the indicator and the same compounds prepared in solution. We have checked TLC in four different systems, HPLC, 500 MHz ^{1}H NMR spectrum of the solution in dimethylsulfoxide, CD spectrum in trifluoroethanolic solution, uterotonic (in vitro) and galactogogic (in vivo) activities in rats. We have found no differences. The properties of other peptides specified in the theoretical part will be presented separately together with their biological activities and/or spectral properties.

Qualitative Continuous Monitoring in Multiple Continuous-Flow Solid-Phase Peptide Synthesis

The synthesis was carried out in polypropylene flow reactors, adjustable for volume, each being initially charged with 400 mg of benzhydrylamine copoly(styrene-1% divinylbenzene) resin (contents of amino group 0.4 meq/g). The detailed synthesis protocol is described elsewhere[4,5]. The concatenated flow reactors were disconnected before the condensation reaction. A polypropylene syringe was charged with 5 ml of a 0.2 M solution of an appropriate hydroxybenzotriazole ester in dimethylformamide and 0.2 ml of a 0.001 M solution of bromophenol blue in dimethylformamide. A part of this solution (2 ml) was injected into each reactor separately; after 5 min the rest of the solution was injected. After the disappearance of blue colour, the reactors were washed with dimethylformamide, re-concatenated, and the synthesis was carried on. A sample of the peptidyl resin was checked by the ninhydrin test; failure to detect free amino groups by bromophenol blue was never observed. The yield and purity of crude peptides are summarized in Table IV.

Quantitative Monitoring

A sample of washed and dried resin (2–5 mg) was loaded into a small vessel with sintered-glass bottom and washed with dimethylacetamide and dichloromethane. Then it was treated with

a solution of bromophenol blue in dimethylacetamide (5% solution, 0.2 ml, 2 × 30 sec) and a saturated solution of bromophenol blue in dichloromethane (0.2 ml, 30 sec). The resin would first turn dark blue and then slowly orange (excess of indicator). The resin was washed carefully with 5% ethanol in dichloromethane until the indicator excess was removed. Elution of the colouring was carried out with 5% diisopropylethylamine in dichloromethane or dimethylformamide (2 ml, or until the beads became colorless), the solution was adjusted to an appropriate volume (25 to 100 ml) with ethanol, and absorbance at 600 nm was read. The substitution was calculated according to the equation:

$$S = A\,V\,/\,91\,800\,m, \qquad (1)$$

where S is substitution of the resin (in mmol/g), A is absorbance of the solution, V is volume (in ml), and m is the amount of the resin sample (in g).

Quantitative Determination of Quaternary Ammonium Groups on the Resin

Samples of the chloromethylated polystyrene resin (100 mg, 0.81 mmol Cl per g) were shaken in a closed vial together with the solution (5%, v/v) of the appropriate base in dichloromethane. After 0.5, 5, 48, and 180 h the resin was filtered and washed with dichloromethane and treated with a 1% solution of bromophenol blue in dimethylacetamide. Excess of the dye was washed out with dichloromethane and dimethylformamide and colour was eluted by a solution of tetraethylammonium chloride in dichloromethane. After the addition of 5 ml 5% DIEA in dichloromethane and adjustment of the volume to 25 or 100 ml (in the cases of higher substitution a further dilution was needed) by ethanol, the absorbance at 600 nm was read and substitution calculated according to Eq. (*1*). The results are given in Table II.

REFERENCES

1. Merrifield R. B.: J. Am. Chem. Soc. *85*, 2149 (1963).
2. Barany G., Merrifield R. B. in: *The Peptides, Analysis, Synthesis, Biology* (E. Gross and J. Meienhofer, Eds), Vol.2, p.1. Academic Press, New York 1980.
3. Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I.: Anal. Biochem. *34*, 595 (1979).
4. Krchňák V., Vágner J., Flegel M., Mach O.: Tetrahedron Lett. *28*, 4469 (1987).
5. Krchňák V., Vágner J., Mach O.: Int. J. Pept. Protein Res., in press.
6. Atherton E., Cameron L., Meldal M., Sheppard R. C.: J. Chem. Soc., Chem. Commun. *1986*, 1763.
7. Gisin B.: Anal. Chim. Acta *58*, 248 (1972).
8. Sarin V. K., Kent S. B. H., Tam J. P., Merrifield R. B.: Anal. Biochem. *117*, 147 (1981).
9. Jošt K., Lebl M., Brtník F. (Eds): *Handbook of Neurohypophyseal Hormone Analogs*. CRC Press, Boca Raton 1987.
10. Hope D. B., Murti V. V. S., du Vigneaud V.: J. Biol. Chem. *237*, 1563 (1962).
11. Barth T., Krejčí I., Kupková B., Jošt K.: Eur. J. Pharmacol. *24*, 183 (1973).
12. Wain-Hobson S., Sonigo P., Danos O., Cole S., Alizon M.: Cell *40*, 9 (1985).
13. Guyader M., Emerman M., Sonigo P., Clavel F., Montagnier L., Alizon M.: Nature *326*, 662 (1987).
14. Krchňák V., Mach O., Malý A.: Anal. Biochem. *165*, 200 (1987).
15. Lebl M., Hruby V. J., Slaninová J., Barth T.: Collect. Czech. Chem. Commun. *50*, 418 (1985).

Translated by the author (V.K.).

Always check for quaternary ammonium groups content before you start the synthesis. Quaternary ammonium groups can be formed also during treatment of the chloromethylated resin with dimethylformamide, especially at elevated temperatures (e.g. during coupling of the first amino acid to the resin). N-Methylpyrrolidone (free of dimethylamine !) is recommended as DMF substitute.

Note for Applied Biosystems synthesizer users: If you want to see the color change, you have to switch off the light behind the vessel, and use program without addition of base to the reaction. BB can be added to HOBt solution (0.001-0.005M).

We wish you success with BB method. Please, let us know your experience (especially if you find problems).