Journal of Chromatography, 266 (1983) 401–408
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 030

DERIVATIZING REAGENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETECTION OF PEPTIDES AT THE PICOMOLE LEVEL

JAMES L. MEEK
Laboratory of Preclinical Pharmacology, National Institute of Mental Health, St. Elizabeths Hospital, Washington, DC 20032 (U.S.A.)

SUMMARY

The sensitivity of detection of peptides by measurement of absorbance at 210 nm is too low to allow analysis of neuropeptides in tissue extracts. To improve sensitivity, derivatives were sought which would be small, hydrophilic, easily detected electrochemically and would react cleanly with multiply substituted peptides giving single derivatives. The most promising compounds were 3,6-dinitrophthalic anhydride (DPNT) and 2-carboxy-4,6-dinitrophthalic anhydride (CDNFB). DNPT derivatives can be detected electrochemically by reduction at −0.24 V. CDNFB derivatives can be measured by absorbance at 360 nm. These derivatives allow improvements in detection of a simple peptide of 50-fold (CDNFB) to 500-fold (DNPT).

INTRODUCTION

High-performance liquid chromatography (HPLC) has proven useful for the separation and preparative isolation of peptides. However, the poor sensitivity of detection of peptides by absorbance measurement at 210 nm has made it difficult to use HPLC for the direct measurement of underivatized peptides in tissue extracts. Derivatization of peptides (either pre- or post-column) should be useful in increasing the sensitivity of detection. Requirements for a suitable pre-column derivatizing reagent are stringent, and include ease of detection, derivative stability, ease of removal of excess reagent and byproducts and, most importantly, a reaction that is either quantitative or negligible with the many functional groups present on peptides. The experiments described in this paper are attempts to find good derivatizing reagents for primary amino groups that would improve sensitivity of detection by electrochemical or absorbance detectors.

METHODS AND MATERIALS

The HPLC apparatus consisted of two Altex Model 110 pumps controlled[1] by an AIM-65 microprocessor operating in FORTH language, a Rheodyne 7125 sample injector, a Bio-Rad ODS-25 column (250 × 4 mm I.D., 10 μm), an Altex–Hitachi variable-wavelength detector, and a Bioanalytical systems LC2A detector with dual

glassy carbon electrodes. To reduce oxygen interference when the electrochemical detector was in use, input lines to the pumps, and connections to the detector were made of stainless-steel tubing. The mobile phase (degassed by bubbling with helium)[2] was pumped through the column for at least 2 h prior to sample injections. Chemicals were purchased from Sigma, Aldrich or Pierce. Lucifer vinyl sulfone was a gift of W. W. Stewart, Bethesda, MD, U.S.A.

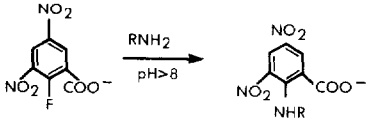
Relative lipophilicity was determined by chromatographing Val-Val and its derivatives with a linear gradient of 0.1 *M* NaH_2PO_4–0.2% H_3PO_4–0% acetonitrile at time of injection to 60% acetonitrile at a rate of 0.75%/min. The retention of the derivatives was compared with that of the underivatized peptide.

Oxidation and reduction potentials were determined with a Tacussel polarograph connected to the Bioanalytical Systems flow cell. Mixtures of Val-Val plus the reagents described below or a blank consisting of only the reagents were chromatographed. When the Val-Val derivative was eluted, the flow was stopped and a differential pulse polarogram was obtained. Pulses of 25 mV were superimposed on a linear voltage ramp of 10 mV/sec.

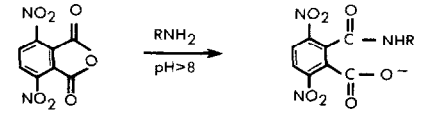
Reaction rates of aryl halides were determined spectrophotometrically at 400 nm. The halides (10 m*M*) and peptides or N-protected amino acids (0.1 m*M*) were treated in phosphate or borate buffers pH 7–11. Measurement of absorbance *vs.* a reagent blank was made at intervals with a Beckman DB spectrophotometer. Rates were estimated[3] by plotting log $(A_{inf} - A)$ *vs.* time, where A_{inf} is the absorbance at infinite time. Extinction coefficients were determined by derivatizing Val-Val with a large excess of reagent, isolating the derivative by HPLC and measuring the absorbance of the peak with a Cary Model 219 spectrophotometer.

Synthesis of reagents

3,6-Dinitrophthalic anhydride (DPNT) (Fig. 1) was prepared by extraction and dehydration of 3,6-dinitrophthalic acid pyridine salt (Sigma). The salt (0.5 g) was suspended in 0.5 ml of 6 *M* hydrochloric acid and extracted twice with 5 ml of diethyl ether. The combined ether phases were washed with 0.5 ml water and evaporated to dryness. The residue (340 mg, m.p. 197–199°C, lit.[4] = 200°C) was dehydrated with



2-CARBOXY-4,6-DINITRO FLUOROBENZENE



3,6-DINITROPHTHALIC ANHYDRIDE

Fig. 1. The formation of CDNFB and DNPT derivatives of peptides.

0.2 ml acetic anhydride at 100° for 10 min, then evaporated to dryness in a vacuum centrifuge at room temperature (yield, 285 mg (80%); m.p., 143–144°C, lit.[4] 145°C).

2-Carboxy-4,6-dinitrofluorobenzene (CDNFB) (Fig. 1) was prepared by nitration of *o*-fluorobenzoic acid (Sigma) by methods analogous to those of Ullman[5] for the preparation of the corresponding chloro derivative. A fume hood was required because of the toxic vapors evolved. A 10-ml volume of fuming sulfuric acid (30% SO_3) in a 25-ml Erlenmeyer flask was cooled in an ice bath on a magnetic stirrer/hot plate. Then 5 ml of concentrated nitric acid (70%) were added, dropwise at first. The temperature rise was limited to 40°C to minimize the formation of nitrosylsulfuric acid. After all the nitric acid had been added, the ice bath was removed and 2 g of *o*-fluorobenzoic acid were added. The mixture was heated with stirring to 145°C for 45 min. Progress of the reaction was monitored by HPLC with detection at 220 nm and a mobile phase of 0.1% trifluoroacetic acid in 25% acetonitrile at a flow-rate of 2 ml/min. The retention times of the starting material, mononitro derivative and product were 3.6, 4.2, and 4.9 min, respectively. At the completion of the reaction (less than 1% starting material or mononitro derivative) the flask contents were cooled to room temperature and poured over 60 g of crushed ice. The precipitate was collected by suction filtration, washed twice with 30 ml of ice water and dried in a vacuum (yield, 2.2 g of colorless crystals; m.p., 197–198°C, lit.[6] = 200°C, decomp.)

5-Nitrosulfobenzoic anhydride was prepared by nitration of *o*-sulfobenzoic cyclic anhydride (Aldrich) with fuming nitric acid[7].

3,5-Dinitrophthalic anhydride was prepared by nitration of *o*-toluic acid and subsequent nitric acid oxidation of the product at 170°C in a sealed tube[8].

2-Nitro-4-sulfofluorobenzene potassium salt was prepared by sulfonation of 2-nitrofluorobenzene[9] or by sulfonation of fluorobenzene[10] with fuming sulfuric acid at 60°C for 1 h. Then, without isolation, the cooled mixture was nitrated by addition of solid potassium nitrate in small portions. The mixture was then poured into ice. The product was precipitated by addition of excess solid potassium chloride. Unsuccessful attempts were made under more severe conditions to produce 4-sulfo-2,6-dinitrofluorobenzene (which would be superior to the mononitro compound in reaction rates and absorbance characteristics). Although some of the dinitro compound was apparently formed by nitration with potassium nitrate at 90°C, large amounts of dinitrofluorobenzene were formed as a side-product, and precipitation of the desired compound with potassium chloride did not occur.

4-Carboxy-2-nitrofluorobenzene was prepared by room-temperature nitration of 4-fluorobenzoic acid with conc. sulfuric acid–conc. nitric acid (4:1)[5]. Attempts to produce the dinitro derivative under more severe conditions (fuming sulfuric acid, higher temperatures, potassium nitrate) were unsuccessful.

Trifluoroacetic acid methyl ester was prepared by adding the acid to methanolic hydrochloric acid (formed by slow addition of acetyl chloride to anhydrous methanol). The ester was isolated by distillation (b.p., 44°C).

Dihydroxyphenylacetic acid N-hydroxysuccinimide was prepared using dicyclohexylcarbodiimide as a condensing agent[11].

Preparation of derivatives

Val-Val was treated in buffers at pH 7–11 at room temperature up to 50°C depending upon the reactivity of the reagents. Completeness of reaction was de-

termined with HPLC by monitoring the disappearance of the Val-Val peak at 210 nm. Starting conditions for the reactions were as described in the literature:

(1) Reductive amination with pyridoxal phosphate and $NaBH_4$ (refs. 12 and 13).

(2) Oxidative amination with hydroquinone-2-sulfonic acid and $K_3Fe(CN)_6$[14,15].

(3) Arylation with trinitrobenzenesulfonic acid[16], 4-carboxy-2,6-dinitrochlorobenzene[17], 4-sulfo-2-nitrofluorobenzene[9], 4-carboxy-2-nitrofluorobenzene, and 2-carboxy-4,6-dinitrofluorobenzene.

(4) Acylation with "active esters": Bolton–Hunter reagent[18] (*p*-hydroxyphenylpropionic acid-N-hydroxysuccinimide), dihydroxyphenylacetic acid N-hydroxysuccinimide and trifluoroacetic acid methyl ester;

(5) Acylation with acid anhydrides: 3-nitro[19], 3,5-dinitro- and 3,6-dinitrophthalic anhydride 2-sulfobenzoic cyclic anhydride, 3-nitro-2-sulfobenzoic anhydride trifluoroacetic anhydride, trichloroacetic anhydride.

(6) Thiourea formation with 3- and 4-sulfophenyl isothiocyanate[20].

(7) Reaction with Lucifer vinyl sulfone[21].

RESULTS

The relative lipophilicity and ease of oxidation or reduction of a number of derivatives are shown in Table I. Hydrophilic derivatives were sought which would have an $E_{1/2}$ as close to zero as possible. A number of compounds were not oxidizable

TABLE I

REDOX POTENTIALS AND RELATIVE RETENTION OF VALYL-VALINE DERIVATIVES

	Val-Val derivative	*Retention*, Val-Val deriv. / Val-Val*	*Redox potential, $E_{1/2}$ (V)***
1	Pyridoxal phosphate	1.5	NRO***
2	Trichloroacetyl	2.1	−0.90 (Hg/Au) §
3	3,6-Dinitrophthalyl	2.4	−0.24
4	5-Nitrosulfobenzoyl	2.6	−0.67
5	3,4-Dihydroxyphenylacetyl	2.7	+0.55
6	2-Sulfobenzoyl	2.7	NRO
7	Maleyl	2.8	−0.96
8	Lucifer vinyl sulfonyl	2.0	(Fluorescent)
9	3-Nitrophthalyl	3.1	−0.57
10	Trifluoroacetyl	3.2	NRO
11	3,5-Dinitrophthalyl	3.4	−0.41
12	4-Hydroxyphenylpropionyl	3.5	+0.89
13	2-Nitro-4-sulfophenyl	3.7	−0.72
14	2,4,6-Trinitrophenyl	6.1	−0.52

* Phosphate–acetonitrile gradient, pH 2.1, 0.75% min, 1 ml/min.

** Half-wave potential *vs.* Ag/AgCl on glassy carbon.

*** NRO = Not reducible or oxidizable, −1.0 to +1.0 V.

§ (Hg/Au) mercury film on gold working electrode; not reducible on glassy carbon.

or reducible in the range from −1.0 to 1.0 V: the trifluoroacetyl, pyridoxyl phosphate, and sulfobenzoyl derivatives. As expected, ease of oxidation and hydrophilicity increased with number of electron-donating groups (*cf.* compounds 5 and 12). The ease of reduction increases with number of electron-withdrawing groups such as nitro, sulfo, carboxy) (*cf.* compounds 3 and 9, 14 and 13).

The reagent that formed the most easily oxidizable derivative was the active ester of DOPAC (number 5). However, this compound did not react cleanly, possibly due to oxidative breakdown at pH 8 or polymerization due to the nucleophilic character of the ionized phenolic groups. Attempts to perform the reaction at lower pH with acid catalysis[22] were unsuccessful. The oxidation potential for the Bolton–Hunter reagent is too high for maximum sensitivity analysis.

The compound that was most easily reducible and most hydrophilic was the 3,6-dinitrophthalyl derivative (DNPT, derivative 3). DNPT reacted rapidly at pH 8 ($T_{1/2}$ < 5 sec) with a variety of simple peptides. Only single derivatives were formed even when the peptide was multiply-substituted (*e.g.* Lys-Lys-Lys). There was no evidence of racemization. The limit of detection of DNPT-Val-Val by reduction at −0.24 V was 1 pmol, in contrast to 500 pmol for the underivatized peptide by measurement of absorbance at 210 nm. The absorbance of DNPT-Val-Val (ε_{210} = $1.8 \cdot 10^4$ l mol^{-1} cm^{-1}) is not substantially greater than that of Val-Val (ε_{210} = $3.8 \cdot 10^3$ l mol^{-1} cm^{-1}). The reagent is readily soluble in anhydrous acetonitrile, and stable in acetonitrile for several days. Hydrolysis in water is very rapid ($T_{1/2}$ < 0.5 min).

Several aryl halides were examined for potential usefulness as chromophoric derivatizing reagents. These analogues of Sanger's reagent (dinitrofluorobenzene)[23] are easily detected by absorbance measurement. The sulfonated compound (Table II, derivative 13) is the most hydrophilic of the compounds. However, the very low reactivity[9] (overnight reaction was required) is undesirable. The 4-carboxy isomer (derivative 16) prepared with the corresponding chloro compound also reacted very slowly[17]. The 4-carboxy-2-nitrofluorobenzene reaction was rapid, but the derivative (number 17) had the expected low absorbance. Of the reagents tested, the 2-carboxy-dinitrofluorobenzene therefore appeared most promising in terms of reaction rate and absorbance characteristics, even though it is lipophilic at acid pH. The pH *vs.* reaction rate with CDNFB was compared for a compound containing an α-amine (Val-Val),

TABLE II

RETENTION AND ABSORBANCE CHARACTERISTICS OF SEVERAL VAL-VAL DERIVATIVES

Derivatives were chromatographed with a gradient of aqueous 0.1 % trifluoroacetic acid to acetonitrile at 4%/min, flow-rate 2 ml/min. The retention time of underivatized Val-Val was 2.6 min.

Derivative	*Val-Val derivative*	*Retention time (min)*	*Absorbance max. (nm)*	$\varepsilon \cdot 10^{-3}$ *(l mol^{-1} cm^{-1})*
13	4-Sulfo-2-nitrophenyl	9.1	420	4.0
15	2-Carboxy-4,6-dinitrophenyl	12.9	357	10.5
16	4-Carboxy-2,6-dinitrophenyl	12.5	260	18
17	4-Carboxy-2-nitrophenyl	12.0	290	5.0

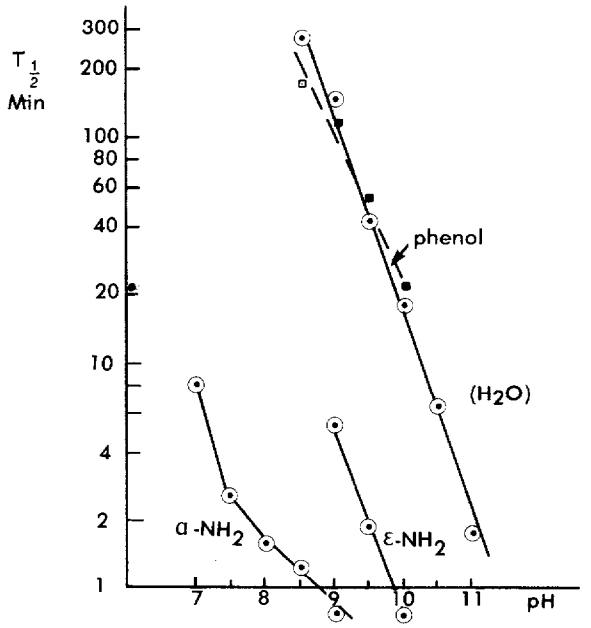


Fig. 2. pH *vs.* reactivity of CDNFB with an α-amine (Val-Val) and ε-amine (N-α-acetyllysine), and a phenol. The pH dependence of hydrolysis of the reagent is also shown.

an ε-amine (N-α-acetyllysine) and a phenol (N-α-acetyltyrosine) as well as the reaction rate of the reagent with water (Fig. 2). The reaction rate increases with pH, and is greatest for the compounds with the lowest pK_a. While the rates differ considerably, the differences are not sufficient to allow one type of group to be derivatized with negligible reaction with the other groups. Large concentrations of reagent and high pH should be used to ensure complete reaction of all groups. In the absence of water, however, reaction of amines occurs much more rapidly than with phenols (Table III). Reaction in methanol may therefore be a method of selectively modifying amines. Another approach to restricting derivatization to amines is to use thiolysis to cleave derivatives of sulfhydryl and phenolic groups without affecting amine derivatives[24]. Reaction of CDNFB with Tyr-Gly-Gly-Phe-Met at pH 11 gave two peaks correspon-

TABLE III

REACTION RATES OF CDNFB IN METHANOL–TRIETHYLAMINE

Pseudo-first-order half-lives were determined with 10 m*M* CDNFB, 40 m*M* triethylamine and 0.1 m*M* peptide or amino acid in methanol at room temperature. The half-life of CDNFB (10 m*M*) in methanol with triethylamine was also determined using HPLC to separate CDNFB from CDNP-methyl ether.

	Reaction half-life, $T_{1/2}$ (min)
Valyl-valine	3.4
N-α-Acetyl-valine	5.5
N-α-Acetyl-tyrosine	$\geqslant 600$
Methanol	>600

ding to N- and N-,O-substituted derivatives. Addition of mercaptoethanol (0.2 *M* final concentration for 10 min) caused quantitative cleavage of CDNP from the phenol without affecting the CDNP-amine.

Several reagents did not react cleanly to give single derivatives. Trifluoroacetic anhydride in acetonitrile or trifluoroacetic acid gave two peaks with Val-Val, probably due to racemization. Reaction with trifluoroacetic acid methyl ester gave only a single peak. The 3- and 4-sulfophenylisothiocyanates gave very complex chromatograms with a pure standard of Val-Val, and would be of little use without extensive cleanup of the reagents and improvent of reaction conditions. The 2-sulfohydroquinone gave two derivatives with $E_{1/2}$ of +0.2 and +0.6 V at retention times slightly longer than that of the pyridoxal phosphate derivative. These products may represent mono- and disubstituted sulfohydroquinones.

DISCUSSION

HPLC has become the standard laboratory technique for measurement of many neurotransmitters and their metabolites, including catecholamines, indoleamines and amino acids. For these compounds, HPLC has the advantages of ease of separation and sensitivity of detection. The poor sensitivity of detection of neuropeptides has limited the use of HPLC in peptide studies to a role as a separation tool because of the low amounts (< 100 pmol) of peptides present in tissue extracts. Derivatization (either pre- or post-column) should be useful for increasing the sensitivity of detection. Many possible reagents are known[25], not only from analytical methods but also from studies of peptide synthesis, peptide sequencing, enzyme modification, etc. Since amine groups are partially responsible for tailing of peaks in HPLC, amine-directed reagents were chosen for this study. Small hydrophilic derivatives were desired so that the effects of the added group on retention would be small. Both electrochemical and absorbance detection was considered. The former is generally more sensitive, but less widely available and less convenient (especially in the reductive mode where oxygen must be excluded). Chang[26] proposed a reagent that strongly absorb visible light, dimethylaminoazobenzene isothiocyanate (DABITC). However, this reagent is so lipophilic that when the peptide contains more than two reactive groups the derivative becomes water-insoluble. Also, recovery is low (30–70%), possibly due to breakdown. Chang claimed a 2-pmol limit of detection with DABITC in absorbance measurements. Although absorbance is usually not considered an ultrasensitive technique, it can be when the compound has a high extinction coefficient at a high wavelength. At high wavelengths (>250–300 nm), baseline disturbances due to mobile-phase impurities are minimal, and the very stable baseline of fixed-wavelength or tungsten variable-wavelength lamps allows full-scale measurements at 0.01 a.u. with little noise.

A major requirement of a reagent is to produce an easily detected single derivative. Reaction with fluorescamine[27] allows use of fluorescence measurements of UV-opaque mobile phases, but the sensitivity is not better than with UV detection at 210 nm. Pre-column derivatization with fluorescamine has been employed, but the peak shape is poor, and multiple peaks have been observed, even with single amino acids, probably due to racemization or internal lactonization. *o*-Phthalaldehyde (OPA) reacts with both amino acids and peptides, but the adduct with peptides has too low a

quantuum yield of fluorescence to allow sensitive detection by fluorescence. Sensitivity with OPA can be improved by post-column partial hydrolysis with barium hydroxide at high temperatures and pressures, but the improvement is not sufficient to make the technique attractive. Derivatives must not only be easily detectable, but also be stable and quantitatively formed. Dns derivatives, although attractive in terms of fluorescence, are unlikely to be of use for complex peptides[28].

In conclusion, the reagents described here should allow an improvement in sensitivity of detection of peptides of 50- to 500-fold. Whether such reagents will allow direct estimation of neuropeptides in tissue must await further work on pre- and post-derivatization cleanup steps.

REFERENCES

1 J. L. Meek and Z. Rosetti, *J. Chromatogr.*, 211 (1981) 15.
2 S. R. Bakalyar, M. P. T. Bradley and R. Honganen, *J. Chromatogr.*, 158 (1978) 277.
3 J. F. Bunnett and J. J. Randall, *J. Amer. Chem. Soc.*, 80 (1958) 6020.
4 T. Momose and M. Torigoe, *J. Pharm. Soc. Japan*, 71 (1951) 977; *C.A.*, 46 (1952) 1921b.
5 F. Ullman, *Ann. der Chemie*, 366 (1909) 79.
6 H. Goldstein and A. Giddey, *Helv. Chim. Acta*, 37 (1954) 1121.
7 M. B. Stubbs, *Amer. Chem. J.*, 50 (1913) 194.
8 Eder and Widmer, *Helv. Chim. Acta*, 6 (1923) 976.
9 R. P. Carty and C. H. W. Hirs, *J. Biol. Chem.*, 243 (1968) 5244.
10 H. P. Schultz, *Org. Synth. Coll.*, 4 (1963) 364.
11 J. Rudiger and U. Ruegg, *Biochem. J.*, 133 (1973) 529.
12 C. Turano, P. Fasella, P. Vecchini and A. Giartosio, *J. Chromatogr.*, 14 (1964) 201.
13 H. W. Lange, N. Lustenberger and K. Hempel, *Z. Anal. Chem.*, 261 (1972) 337.
14 H. W. Wanzlick, *Newer Methods of Preparative Organic Chemistry*, Vol. 4, Verlag Chemie, Weinheim, Academic Press, New York, London, 1968, p. 138.
15 K. Lorentz and B. Flattner, *Anal. Biochem.*, 38 (1970) 557.
16 A.-C. Spadano, W. Draghetta, S. Nassif del Lama, A. C. Camargo and L. J. Greene, *Anal. Biochem.*, 96 (1979) 317.
17 D. L. Brautigan, S. Ferguson-Miller and E. Margoliash, *J. Biol. Chem.*, 253 (1978) 130.
18 A. E. Bolton and W. M. Hunter, *Biochem. J.*, 133 (1973) 529.
19 Th. Wieland, Chr. Birr and H. Wissenbach, *Angew. Chem., Int. Ed. Engl.*, 8 (1969) 764.
20 F. E. Dewlet and F. R. N. Gurd, *Anal. Biochem.*, 76 (1976) 530.
21 W. W. Stewart, *Nature (London)*, 292 (1981) 17.
22 Nakamizo, *Bull. Chem. Soc. Japan*, 42 (1969) 1071.
23 F. Sanger, *Biochem. J.*, 39 (1945) 507.
24 S. Shaltiel, *Biochem. Biophys. Res. Comm.*, 29 (1967) 178.
25 G. E. Means and R. E. Feeney, *Chemical Modification of Proteins*, Holden-Day, San Francisco, CA, 1971.
26 J. K. Chang, *Biochem. J.*, 199 (1981) 537.
27 M. Rubinstein, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4972.
28 K. Narita, H. Matsuo and T. Nakajima, in S. B. Needleman (Editor), *Protein Sequence Determination*, Springer, Berlin, Heidelberg, New York, 1975, p. 42.