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Note

Reversed-phase liquid chromatography of biotin-labelled nucleotides

A new class of markers in molecular biology

V. TOENGES* and A. REICHERT
Institut für Anthropologie, Abteilung Humangenetik, Johannes Gutenberg-Universität, Saarstrasse 21, 6500 Mainz (F.R.G.)
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Often in molecular biology the problem arises to detect, localize or isolate nucleic acids, even when they are present in extremely small amounts. Up to now in most cases radioactive isotopes (^{3}H, ^{14}C, ^{32}P, ^{125}I) have been used as markers. The tracer technique is a very sensitive method, but there are several disadvantages that have led to a search for alternative labels. Safety precautions have to be observed during storage, preparation, usage and disposal of radioactive material. Secondly, radioactive substances are often very unstable and thus have a limited life-span. Thirdly, the resolving power of radioactive labelled substances is not satisfactory when nucleic acids have to be precisely localized, *e.g.* in distinct regions of chromosomes.

One alternative method was first presented by Langer *et al.*[1–3]. They described a direct labelling of nucleic acids by a haptene that can be visualized immunologically or histochemically: biotin was covalently linked to purine or pyrimidine bases via a linker arm. The modified nucleotides were subsequently used for enzymatic synthesis of DNA or RNA.

In our laboratory we prepared six biotin-labelled nucleotides with different linker arms and tested their usage as markers for RNA molecules[4].

EXPERIMENTAL

Materials

8-Aminohexylaminoadenosine 5′-triphosphate N^6-(6-aminohexylcarbamoylmethyl)adenosine 5′-triphosphate, aminocaproic acid, biotin, CTP, dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide and UTP were purchased from Sigma (München, F.R.G.). Mercuric acetate and potassium tetrachloropalladiate (K_2PdCl_4) were from Fluka (Neu-Ulm, F.R.G.). DEAE Sephadex A-25 was a product from Pharmacia (Freiburg, F.R.G.). All reagents were of analytical grade. Dimethylformamide and DCC were redistilled before use.

Methods

Synthesis of biotinylated nucleotides. The allylamine linker arm was formed by

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dissolving 1 m*M* UTP or CTP in 100 m*M* sodium acetate (pH 6.0) and treating the solution with 5 m*M* mercuric chloride for 4 h at 50°C. After cooling on ice, 9 m*M* lithium chloride was added and the excess of mercuric chloride was repeatedly extracted with ethyl acetate. The mercurated derivatives were precipitated with cold ethanol, centrifuged and washed with ethanol and diethyl ether. Then 0.5 m*M* of the crude mercurated nucleotides in 25 ml of sodium acetate (100 m*M*, pH 5.0) were combined with 6 m*M* allylamine in 3 ml of 4 *M* sodium acetate. The reaction was started by the addition of 0.5 m*M* K_2PdCl_4 in 4 ml of water and was kept at room temperature for 24 h. The black mixture was filtered through a 0.45-μm membrane to remove metallic precipitates. The yellow solution was diluted 1:5 with water, applied to a DEAE Sephadex A-25 column and eluted by a linear gradient of ammonium carbonate (800 ml, 0.1–1 *M*, pH 8.6). Fractions of 5 ml were collected in a

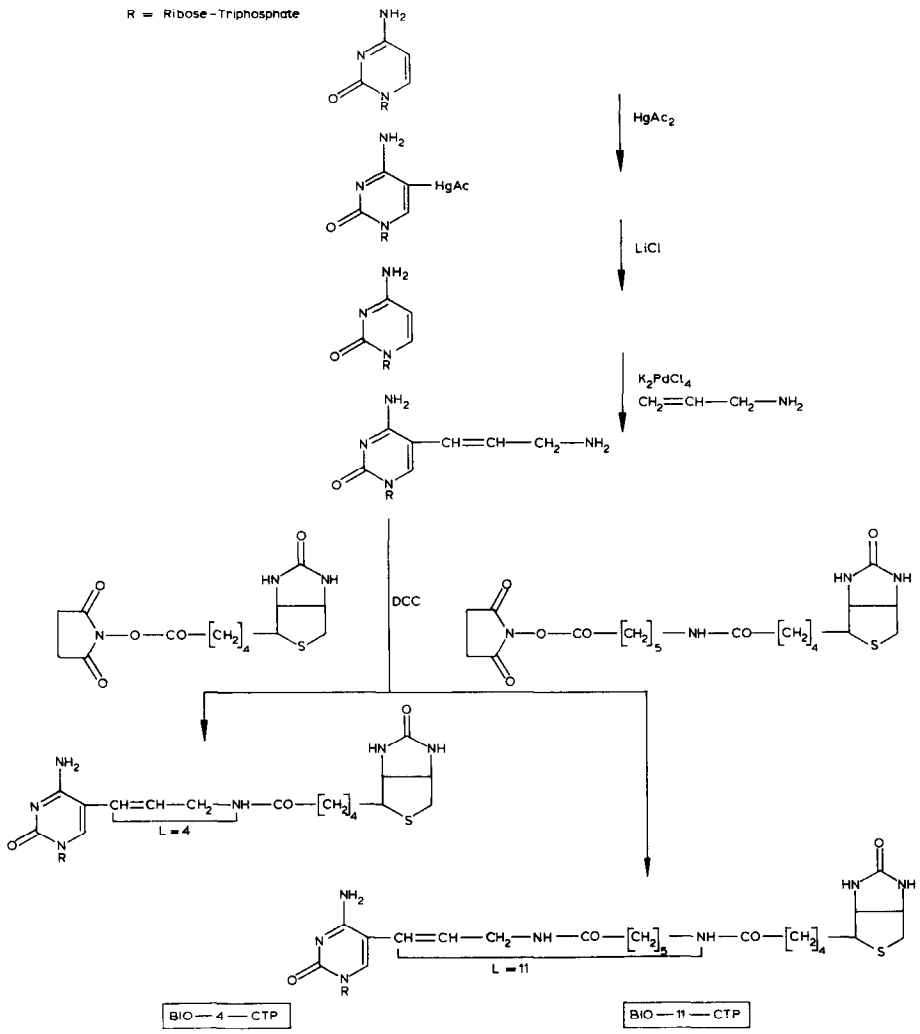

Fig. 1. Synthesis of biotinylated pyrimidines.

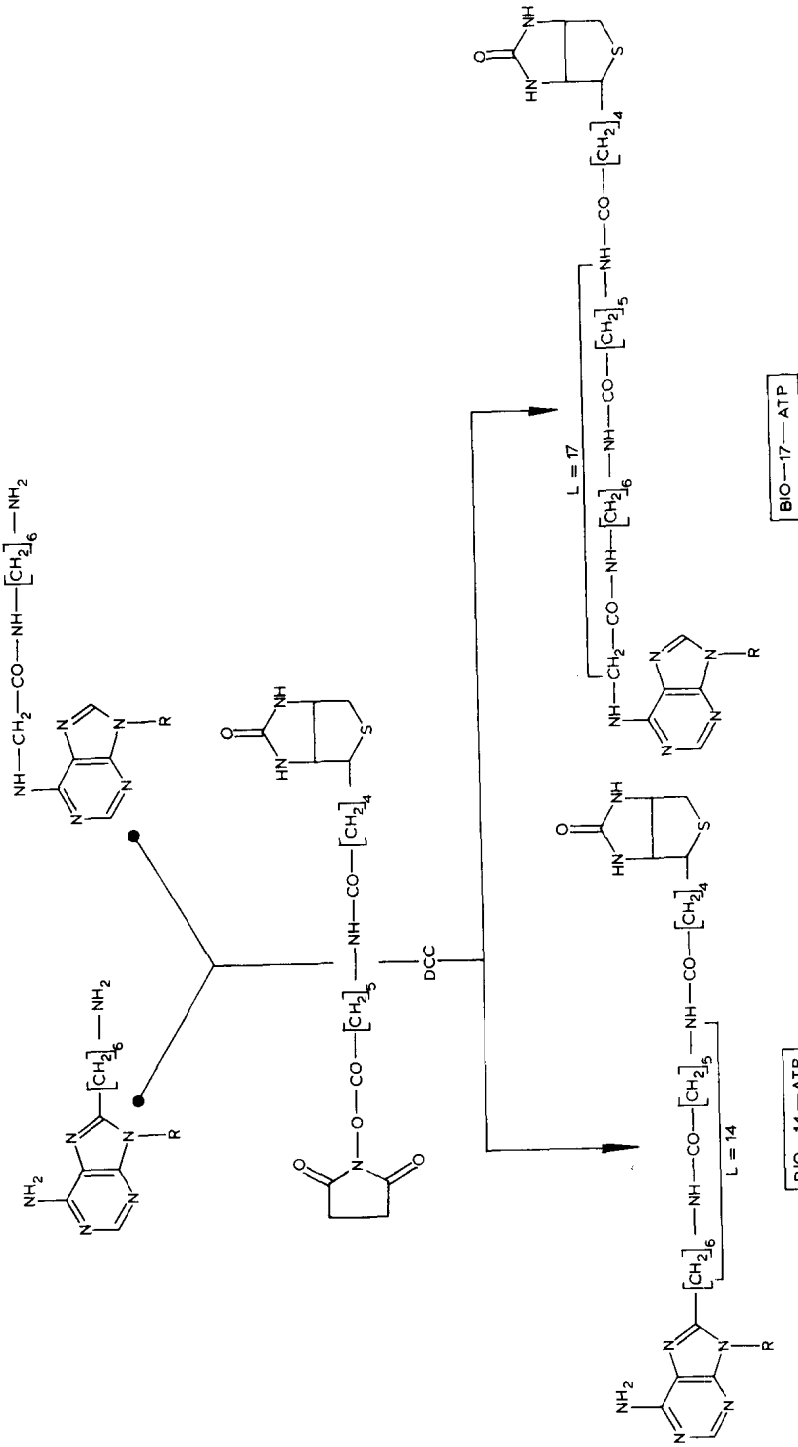

Fig. 2. Synthesis of biotinylated ATP derivatives.

LKB fraction sampler. The UV absorption was registered at 278 nm. The products were desalted by repeated rotary evaporation or by cation-exchange treatment. Biotinyl-N-hydroxysuccinimide ester was prepared from biotin according to Heitzmann and Richards[5]. Caproylamidobiotin-N-hydroxysuccinimide ester was synthesized by the method of Leary *et al.*[6]. Then 0.1 m*M* nucleotide [allylamino-UTP/allylamino-CTP/8-aminohexylaminoadenosine 5'-triphosphate/N^6-(6-aminohexylcarbamoyl-

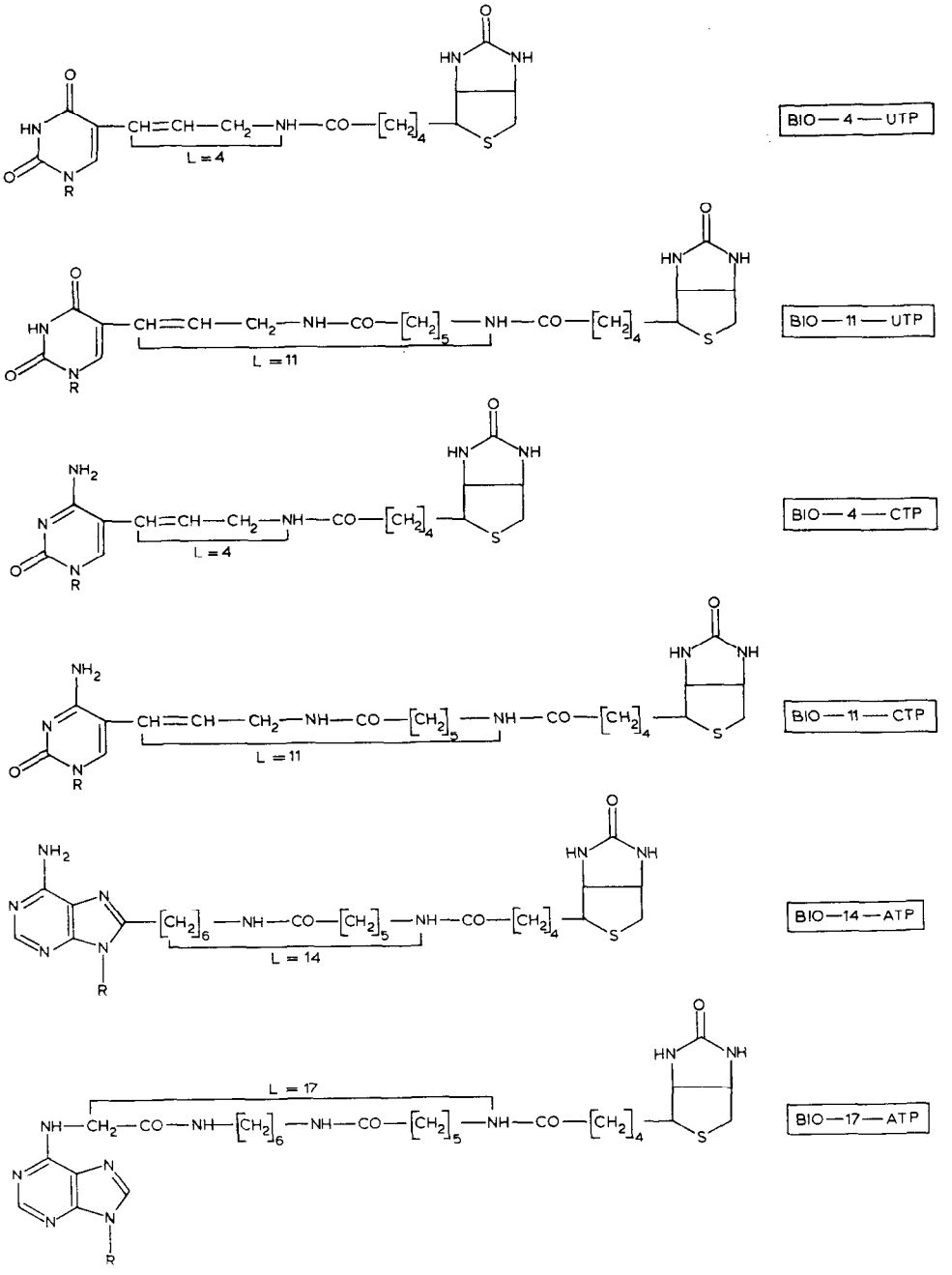

Fig. 3. Synthesized biotin-labelled nucleotides.

methyl)adenosine 5'-triphosphate] in 2 ml of 0.1 *M* sodium borate buffer (pH 8.5) were treated with 0.1 m*M* N-hydroxysuccinimide ester (biotinyl-N-hydroxysuccinimide or caproylamidobiotin-N-hydroxysuccinimide) in 0.2 ml of dimethylformamide for 4 h at room temperature. The whole sample was applied to a Sephadex A-25 column and the products were eluted by a 600 ml linear gradient of ammonium carbonate (0.3–1.2 *M*, pH 7.5). The desired products were desalted by repeated rotary evaporation or by ion-exchange chromatography. All products were checked for their biotin content by the method of McCormick and Roth[7]. Each substance showed the typical red-orange colour when treated with *p*-dimethylaminocinnamaldehyde in ethanolic sulphuric acid.

Characterization of biotinylated nucleotides by reversed-phase high-performance liquid chromatography (HPLC). All synthesized triphosphates were dissolved in 20 m*M* Tris–HCl (pH 7.5) and adjusted to a final concentration of 1 m*M*. A Knauer high-performance liquid chromatograph was used for the separation of nucleotides. The system consisted of a Knauer pump type 64, a Knauer photometer type 97, which registered at 250 nm, a Rheodyne sample injector and a Knauer 250 mm stainless-steel column packed with LiChrosorb RP-18. The sample size was 10 μl per injection. The standard eluent was methanol–0.5 *M* KH_2PO_4 (pH 3.3) (30:70). The flow-rate was either 1 or 1.5 ml/min.

RESULTS AND DISCUSSION

Six biotin-labelled nucleotides were synthesized: Bio-4-UTP, Bio-4-CTP,

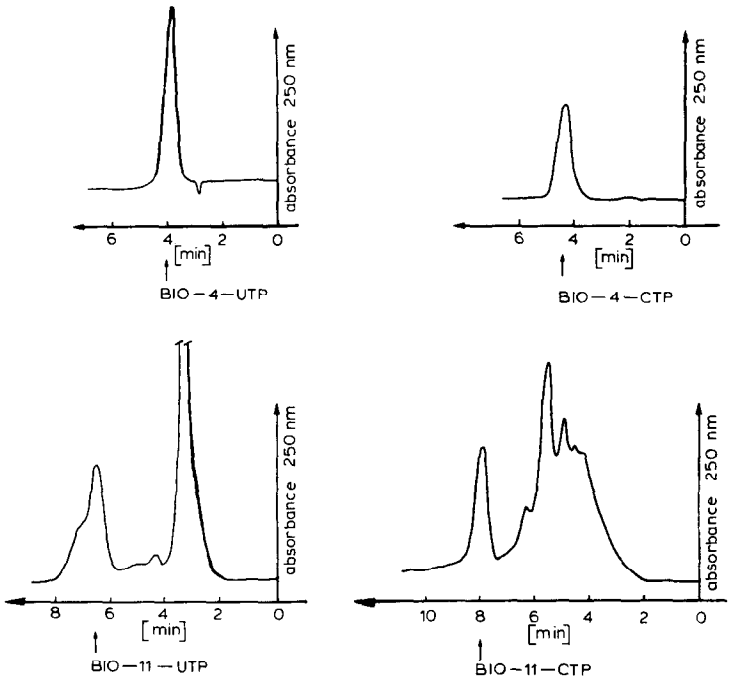

Fig. 4. HPLC elution profiles of biotinylated pyrimidines. For conditions, see Table I.

TABLE I

CHARACTERIZATION OF BIOTINYLATED TRIPHOSPHATES

Synthesized nucleotides	*$(NH_4)_2CO_3$ concentration eluting the product from a Sephadex A 25 column*	*Absorption (nm)*		*Retention time (min)**
		Maximum	*Minimum*	
Bio-4-UTP	0.31–0.34 *M*	240, 290	262	3.9
Bio-11-UTP	0.48–0.54 *M*	240, 288	228, 274	6.5
Bio-4-CTP	0.30–0.34 *M*	272	255	4.3
Bio-11-CTP	0.48–0.56 *M*	242, 294	226, 268	8.0
Bio-14-ATP	0.46–0.54 *M*	278	242	11.9
Bio-17-ATP	0.44–0.51 *M*	268	226	13.5

* On a LiChrosorb RP-18 column; eluent, methanol–0.5 *M* potassium dihydrogen phosphate (pH 3.3) (30:70); flow-rate, 1 ml/min.

Bio-11-UTP, Bio-11-CTP, Bio-14-ATP and Bio-17-ATP (Figs. 1 and 2). The modified triphosphates differed in the base, the length of the linker arms and the position where the linker was attached to the purine or pyrimidine (Fig. 3).

Bio-4-UTP and Bio-4-CTP eluted from the LiChrosorb RP-18 column after 3.9 and 4.3 min (Fig. 4). Both substances were homogeneous and showed no sig-

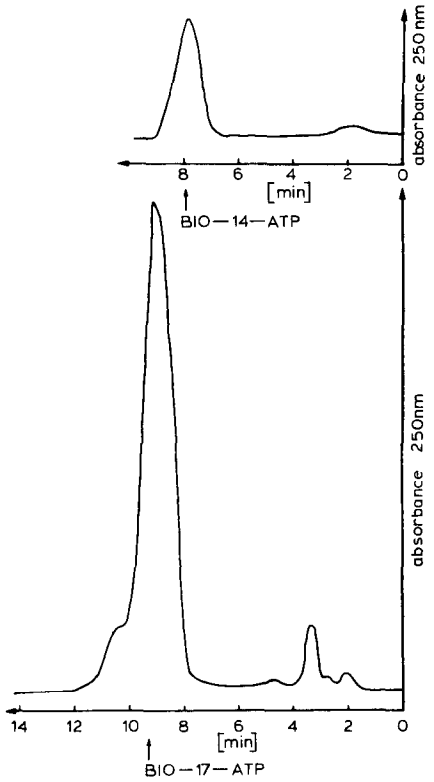

Fig. 5. HPLC elution profiles of biotinylated ATP-derivatives. Conditions as in Table I, except flow-rate (1.5 ml/min).

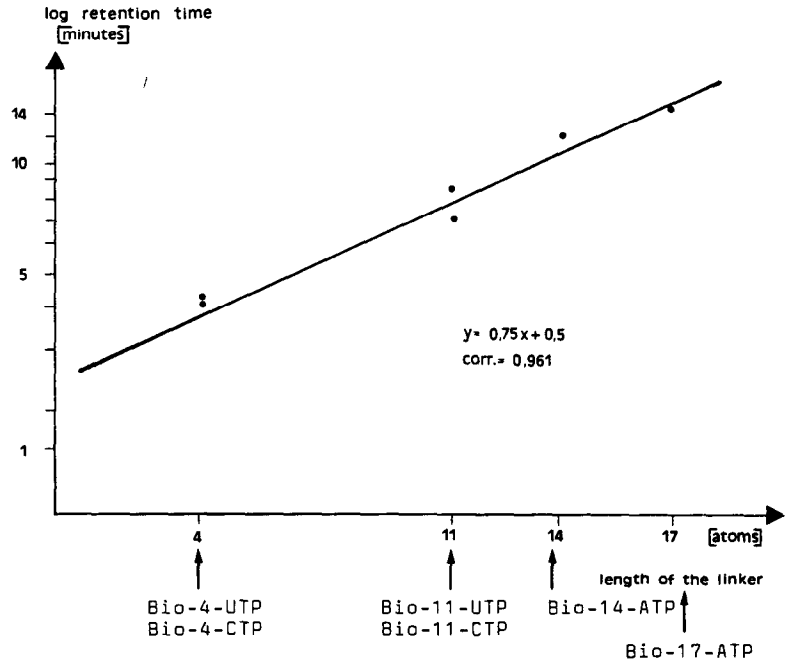

Fig. 6. The homologous series of biotin-labelled nucleotides; a function of the length of the linker arm.

nificant impurities. The caproylamidobiotin-containing derivatives Bio-11-UTP and Bio-11-CTP could be eluted within 6.5 and 8.0 min from the same column. Both preparations were contaminated by several as yet unidentified by-products. For further use, these derivatives must be purified on a preparative HPLC column.

In the mobile phase we used a high salt concentration to reduce the dissociation of the triphosphates. This effect could not be completely controlled, as can be seen in the elution profiles of Bio-11-UTP and Bio-17-ATP (Figs. 4 and 5). Both diagrams showed a shoulder caused probably by the dissociation of the phosphate groups. Increasing the salt concentration would minimize this effect but simultaneously would give rise to severe problems of practical usage (crystallization of the buffer on the column, higher pressure, etc.).

Synthesized products of the same homologous series, where the members differ primarily by the length of the linker and where the influence of different bases is neglected, show a linear relationship between the logarithm of the retention time and the length of the linker arm (Fig. 6).

In further experiments the six triphosphates will be used as markers for RNA molecules and their usefulness will be documented[4].

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