MOLECULAR BIOLOGICAL PROBLEMS IN THE CREATION OF DRUGS AND STUDY OF THE MECHANISM OF THEIR ACTION

Co – C-CORRINOIDS – DERIVATIVES OF PSEUDO FORMS OF VITAMIN B_{12} – AS INHIBITORS OF CORRINOID ENZYMES

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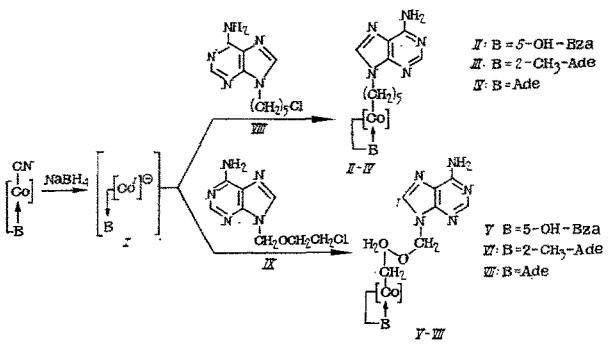
The approach of chemical modification of coenzymes is creating broad prospects for the search for biologically active substances in general and drugs in particular. By modifying the structure of the coenzyme it is possible to obtain a substance retaining various degrees of cofactor activity, as well as inhibitors of enzymes, and thereby, when necessary, to sufficiently specifically modify the activity of the enzymes in the treatment of diseases. Despite the fact that the modification of cobalamin coenzymes has not yet led to such results, the search for drug compounds in the series of analogues of cobalamin coenzymes is urgent and promising.

It is known [1] that the main contribution to the binding of analogues of corrinoid coenzymes to protein is made by corrin tetradentate and nucleotide ligands. Natural analogues of vitamin B_{12}, modified on the α-ligand, are Co α-[α-(5-hydroxybenzimidazolyl)]-Coβ-cyanocobamide (factor III), Co α-[-α(2-methyladen-9-yl)]-Coβ-cyanocobamide (factor A), and Co α-[α-(aden-9-yl)]-Coβ-cyanocobamide (pseudo-B_{12}), which accompany true vitamin B_{12} in the process of its microbiological synthesis.

It has been shown [2] that the coenzyme form of factor III retains catalytic activity in two cobalamin-dependent enzymatic reactions: glycerol dehydratase and glutamate mutase, but in this case the ability to bind to the apoenzyme is higher for it than for adenosylcobalamin (AdoCbl). Moreover, the analogues of AdoCbl that we obtained earlier [3], modified in the riboside portion of the β-ligand, proved to be powerful competitive inhibitors of the glycerol dehydratase reaction. For a search for new biologically active compounds in the series of vitamin B_{12} derivatives, it seemed interesting to us to synthesize analogues with the same modifications in the β-ligand, but based on natural factors of vitamin B_{12}.



Bza stands for benzimidazole, Ade for adenine.

In this work we describe the synthesis and physicochemical properties of the following compounds: Coα-[α-(5-hydroxybenzimidazolyl)]- Coβ-[5-(aden-9-yl)-pentyl] cobamide (II), Coα-[α-(2-methyladen-9-yl)]-Coβ-[5-(aden-9-yl)pentyl]cobamide (III), Coα-[α-(aden-9-yl)]-Coβ-[5-(aden-9-yl)pentyl]cobamide (IV), Coα-[α(5-hydroxybenzimidzolyl)]-Coβ-[(aden-9-yl)methoxyethyl]cobamide (VI), and Coα-[α-(aden-9-yl)]-(aden-9-yl)]-Coβ-[(aden-9-yl)methoxyethyl]cobamide (VII).

Compounds II-VII were produced by the reactions of reduced corrinoids (I) with the following alkylating agents: 9-(5'-chloropentyl)adenine (VIII) or 9-(1'-methoxy-2 -chloroethyl)adenine (IX). The synthesis and properties of compounds VIII and IX were described in [4]. The reaction was conducted under conditions

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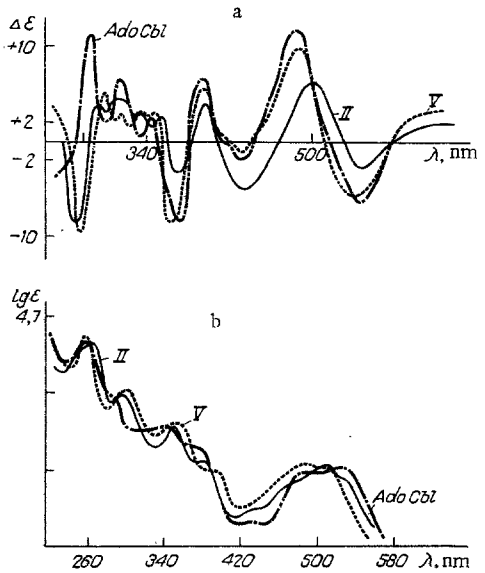

Fig. 1. CD spectra (a) and absorption spectra (b) in water of Co α-[α-(5-hydroxybenzimidazolyl)]-Coβ-[(aden-9-yl)methoxyethyl]cobamide (V), Co α-[α-(5-hydroxybenzimidazolyl)Coβ-[5-(aden-9-yl)-pentyl]cobamide (II), and adenosylcobalamin (AdoCbl).

analogous to the production of AdoCbl and alkyladenine and methoxyethyl analogues of the cobamide coenzyme, synthesized previously [3]. The corrinoids were isolated from the reaction solution by phenol extraction and by chromatographic separation on columns with carboxymethylcellulose in the H^+ form. Eluates from the column were lyophilized.

In the production of compounds II-VII, the formation of a small quantity of organocorrinoids, which were similar in their spectral properties and electrophoretic mobility to alkylcorrinoids, was observed. We did not demonstrate the structure of these compounds, but by analogy with the production of alkyladenyl analogues of vitamin B_{12} it can be assumed that these side products did not contain adenine in the β-ligand [3].

The synthesized analogues of factors AdoCbl are individual in chromatography in various solvent systems and electrophoresis at neutral and acid pH values. Compounds II-VII, immobile in electrophoresis in a neutral solvent system, acquired two positive charges in acid medium, which confirmed the presence in the molecule not only of an α-ligand capable of protonation but also of an adenine fragment (Table 1).

The absorption spectrum of compound V in the UV and visible regions is similar to the spectrum of AdoCbl (Fig. 1). On the contrary, in the spectrum of the analogue II (see Fig. 1) in the visible region, only one maximum was observed at 510 nm. In contrast to organocorrinoid V, the absorption spectrum of the analogue II resembles the spectrum of alkylcobalamins [5] and differs from the latter only by the presence of an intense maximum in the region of 263 nm, probably due to the contribution of the adenine chromophore. In the visible region of the absorption spectra of compounds III, IV, VI, and VII, which are alkyladenyl analogues of factor A and pseudo-B_{12} (Fig. 2), a hypsochromic shift of the absorption maxima into the region of 460 nm was observed, which is characteristic of organocorrinoids in which there is no coordination of the cobalt atom to the α-ligand. An analogous fact is known for coenzyme forms of factor A and pseudo-B_{12} [6].

The absence of coordination of the cobalt atom with the α-ligand in compounds III, IV, VI, and VII is confirmed by the data of the CD spectra: The appearance of intense maxima is observed in the region of 328-331 nm (see Fig. 2) [7].

The presence of a Co–C bond in the compounds obtained was demonstrated by the appearance of low-intensity maxima in the region of 375-380 and 340 nm. All the analogues obtained are photolabile, which is an additional confirmation of the presence of a Co–C bond in them. During photolysis, the corresponding hydroxy form was produced, which was demonstrated spectrally according to the appearance of an intense maximum in

TABLE 1. Physicochemical Characteristics of the Compounds Obtained

Compound	Yield, %	R_f AdoCbl *		E_{AdoCbl} *		UV spectrum		CD spectrum	
		A	B	C	D	λ_{max}, nm	lg ε	λ_{max}, nm	$\Delta\varepsilon$, $mole^{-1} \cdot cm^{-1}$
II	79,5	0,80	0,84	1,02	—†	263, 299, 352, 380 sh, 510	4,52; 4,29; 2,13; 3,92; 3,91	255, 276, 298, 318, 338, 362, 388, 428, 495, 552	—8,72; +4,10; +4,36; +3,08; +2,97; —4,92; +5,08; —5,34; +6,61; —3,73
III	69,6	0,55	0,92	1,03	—	265, 304, 354 sh, 380 sh, 440, 468	4,50; 4,35; 4,05; 3,94; 3,73; 3,86	257, 303, 332, 360, 381, 420, 460, 513, 575	—18,34; +10,98; +16,47; —6,40; +2,06; —6,86; +0,69; —3,69; +3,89
IV	70,9	0,40	0,95	1,07	—	265, 304, 381, 460	4,32; 4,09; 3,65; 3,59	258, 304, 330, 358, 380, 422, 510, 570	—10,67; +6,51; +9,64; —1,22; +0,84; —3,51; —2,37; +2,06
V	29,1	0,75	0,87	0,95	—	260, 280—300, 338, 374, 482, 524	4,50; 3,77; 4,10; 4,02; 4,01; 3,89	256, 280, 300, 315, 330, 356, 385, 425, 480, 550	—9,23; +5,27; +3,29; +2,97; —3,95; —9,23; +6,26; —0,66; +10,86; —6,92
VI	44,0	0,45	0,83	1,02	—	265, 370, 472, 512	4,55; 4,00; 3,85; 3,82	234, 260, 302, 328, 356, 383, 422, 470, 513, 537, 590	+6,18; —18,29; +4,25; +8,94; —5,96; +2,77; —2,13; +4,25; —5,85; —5,74; +0,85
VII	14,6	0,45	0,83	1,02	—	264, 280 sh, 464, 500	4,70; 4,60; 3,90; 3,84	260; 304, 328, 360, 386, 422, 470, 508, 586	—18,41; +6,00; +11,74; —2,73; +1,47; —4,19; +2,52; —5,03; +2,10

* See Experimental Chemical section.

†The compound is not charged in this solvent system.

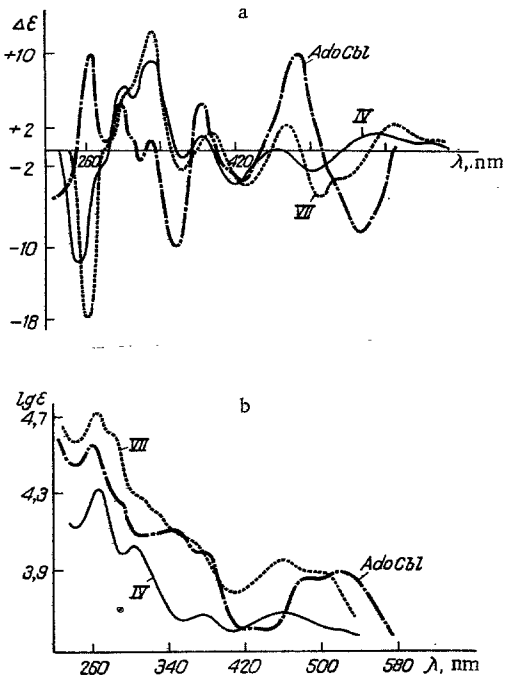

Fig. 2. CD spectra (a) and absorption spectra (b) in water of Co α-[α-(aden-9-yl)]-Coβ-[(aden-9-yl)methoxyethyl]-cobamide (VII), Coα-[α-[aden-9-yl)]-Coβ-[5-(aden-9-yl)pentyl]cobamide (IV), and AdoCbl.

TABLE 2. Clinical Constants of AdoCbl and Its Derivatives in the Glycerol Dehydratase System

Compound	$K_i \cdot 10^{-8}$ M	$K_m \cdot 10^{-8}$ M	V % of AdoCbl	Compound	$K_i \cdot 10^{-8}$ M	$K_m \cdot 10^{-8}$ M	V % of AdoCbl
AdoCbl	—	2,0	100	VI	0,9	—	—
II	0,6	—	—	VII	0,3	—	—
III	1,0	—	—	X	—	3,3*	100*
IV	0,45	—	—	XI	—	1,2*	100*
V	0,25	—	—	XII	—	1,0†	89†

*Values taken from [10].
†Values taken from [2].

the region of 350 nm, and also electrophoretically with a reference standard and by the reaction of cyanation, resulting in the production of dicyanides of compounds II-VII with the characteristic absorption maximum in the region of 368 nm.

The CD spectrum of compounds II and V is characterized by the appearance of a supplementary maximum in the region of 318−315 nm with Δε +3,08 and +2,97, respectively. The increase in the intensity of the positive maximum in the region of 330 nm in the CD spectra of compounds II and V (see Fig. 1) is apparently evidence of a partial cleavage of the bond of cobalt to the α-ligand. Since the CD spectra are the most sensitive to conformational changes, the differences existing in the spectra of the analogues are apparently evidence of certain changes in the conformation of the corrinchromophore, caused by changes in the structure of the β- and α-axial ligands.

The synthesized analogues of AdoCbl were studied in the glycerol dehydratase (EC 4.2.1.30) system. This enzyme catalyzes the irreversible conversion of glycerol, 1,2-propanediol, and ethylene glycol to 3-hydroxypropionic and propionic aldehydes and acetaldehyde, respectively, and exhibits an absolute requirement for AdoCbl and K^+.

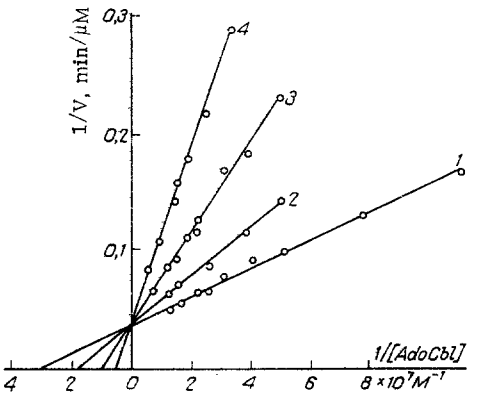

Fig. 3. Dependence of the initial rate of dehydration of 1,2-propanediol on the AdoCbl concentration without inhibitor (1) and in the presence of $3 \cdot 10^{-3}$, $1 \cdot 10^{-2}$, and $3 \cdot 10^{-2}$ μM compound IV (2, 3, 4 respectively) in reciprocal coordinates. Composition of reaction mixture: apoenzyme 0.022 unit, 1,2-propanediol $1 \cdot 10^{-2}$ M, ADH 50 μg, NADH $2 \cdot 10^{-4}$ M. The remaining conditions are described in the Experimental Biological section.

It was established that all six analogues do not possess coenzyme properties. This fact confirmed the conclusion that we had drawn earlier, of an essential role of the ribofuranose fragment of AdoCbl in the manifestation of its coenzyme properties [8]. Then it was shown that in the presence of AdoCbl all six analogues inhibit the rate of conversion of 1,2-propanediol and are competitive inhibitors of the enzyme with respect to AdoCbl (Fig. 3). The values of K_i calculated according to the Dixon method [9] are presented in Table 2. It should be noted that AdoCbl itself possesses an extremely high affinity for the apoenzyme, and the value of its K_m is $2 \cdot 10^{-8}$ M [1].

Earlier we demonstrated [10] that the coenzyme forms of pseudo-B_{12} (compound X) and factor A (compound XI) (see Table 1) entirely retain the properties of cofactors in the system of glycerol dehydratase, and the values of K_m for each of the compounds are $3.3 \cdot 10^{-8}$ and $1.2 \cdot 10^{-8}$ M, respectively.

In a comparison of the values of K_i of compounds II-VII with the values of K_i for AdoCbl and coenzyme forms of pseudo-B_{12} and the investigated factors (see Table 1), it can be seen that the analogues are bound to the apoenzyme better than AdoCbl or coenzyme forms of the factors, but the affinity of compounds V-VII is 7-8 times as great as the affinity of AdoCbl. It must be noted that these analogues are the most powerful inhibitors among the 30 analogues that we studied [1, 2, 8] and can be recommended for biological tests as antagonists of B_{12}-dependent enzymes. From an examination of the atomic models of Dreiding for the coenzyme form of factor III and compounds II and V, as well as from an examination of the atomic models of AdoCbl and its penta- and methoxyethyl derivatives, it follows that the alkyladenine and methoxyethyladenine structures are not so rigid as the sugar residue in AdoCbl. These structures permit the adenine heterocycle to geometrically occupy the same position above the C-ring of the corrin macrocycle as in the coenzyme forms. It can be assumed that the better interaction of the apoenzyme with analogues II-VII is due to the fact that smaller conformational rearrangements of the protein are required for the binding of more flexible acyclic analogues.

Of interest from this standpoint is the recently published work [11], in which it was shown that a large role in ensuring conformational mobility of the corrin ring is played by the structure of the 5'-deoxyadenosyl ligand. Penta- and methoxyethyl fragments, introduced into the structure of this ligand instead of deoxyribose, make the corrin ring of modified AdoCbl conformationally more labile. As a result of this, conditions are not created for the appearance of stress in the interaction of the apoenzyme with corrin, necessary for cleavage of the Co–C bond. This circumstance can apparently explain the absence of coenzyme properties for the investigated analogues.

EXPERIMENTAL CHEMICAL SECTION

The spectra in the UV and visible regions were recorded on a Pye Unicam SP-800 recording spectrophotometer (England), the CD spectra were recorded on a Jasco ORD (UV) CD-5 instrument (Japan) in 0.2 M potassium phosphate buffer, pH 8.0. In the recording of the CD spectra, solutions with a concentration of 25-60 μM and cuvettes with path length 0.2-2 cm were used. The measurement error did not exceed 5% in the region of 350-600 nm and 10% in the region of 220-350 nm. Chromatography was performed on FN-11 paper in the systems: n-butanol—isopropanol—acetic acid—water, 100 : 70 : 1 : 100 (A); sec-butanol saturated with water (B).

Electrophoresis was conducted on FN-11 paper on a UÉF instrument with potential gradient 16.0 V/cm in the solvent systems: 1 N acetic acid pH 2.4 (C); 0.03 M sodium acetate pH 6.7 (D).

The mixture of vitamin B_{12} factors (factor III, factor A, and pseudo-B_{12}) was kindly provided by I. Valu (Gedeon Richter, Hungary).

Separation of the Mixture of Vitamin B_{12} Factors. The mixture of factors is dissolved in a minimum amount of water and applied on a column with carboxymethylcellulose in the H^+ form. The first fraction, containing factor III and traces of vitamin B_{12}, the second fraction containing pseudo-B_{12}, and the third fraction containing acetone are eluted with water and left for 24 h at 5°C. After filtration and drying, the vitamin B_{12} factors indicated above are obtained.

Production of Alkyladenyl and Methoxyethyladenyl Analogues of Vitamin B_{12} Factors. A $1.12 \cdot 10^{-4}$ mole portion of the factor is dissolved in 5 ml of 50% ethanol, and a stream of argon is passed through the system for 30 min for complete displacement of air. Then $20 \cdot 10^{-4}$ mole of $NaBH_4$ in 8 ml of absolute ethanol is added under the same conditions. The solution is mixed for 1 h until the color changes from red-brown to gray-green. To the cobalamin (I) formed, $1.70 \cdot 10^{-4}$ mole of 9-(5-chloropentyl)-adenine (VIII) or $3.19 \cdot 10^{-4}$ mole of 9-(1'-methoxy-2'-chloroethyl)-adenine (IX) in deoxygenated 75% alcohol (8 ml) is added. After mixing for 1 h at room temperature in a stream of argon, the color of the solution gradually changes to red; the solution is evaporated to dryness under vacuum at 30°C, the residue dissolved in 20 ml of water, and the solution subjected to phenol extraction after the addition of 0.2 ml of glacial acetic acid to pH 4.5-5.0. Separation of the reaction solution is performed on a column with carboxymethylcellulose in the H^+ form: The unreacted factor is eluted with water, the hydroxy form of the factor with a 0.2% acetic acid solution, and an organocorrinoid of unknown structure and the analogue sought are eluted with a 2% acetic acid solution.

EXPERIMENTAL BIOCHEMICAL SECTION

In the work we used NADH and a preparation of crystalline alcohol dehydrogenase from horse liver produced by Reanal (Hungary) and 1,2-propanediol from Gee Lawson Chemical Ltd. (England).

The apoglycerol dehydratase preparations were isolated from cell-free extracts and purified by the method of [12]. In the work we used preparations of the apoenzyme freed of glycerol by gel filtration through a column with Sephadex G-25. The glycerol dehydratase activity was determined by the method we developed [13] according to the rate of conversion of 1,2-propanediol to propionaldehyde. The amount of the enzyme catalyzing the conversion of 1 μmole of propionaldehyde per minute in the dehydration of 1,2-propanediol with AdoCbl as a cofactor was taken as the activity unit. The concentration of AdoCbl and its analogues was determined spectrophotometrically at 522 nm for AdoCbl and analogues II and V and at 458 nm for analogues III, IV, VI, and VII, which are characterized by the absence of a coordination bond between the heterocyclic base of the α-ligand and the central Co atom [14].

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INTERACTION OF CERTAIN XENOBIOTICS WITH LIVING CELLS: INVESTIGATION BY THE METHOD OF FLUORESCENT PROBES

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In the body most pharmacological preparations are either bound to the cell membranes or penetrate through them to their "targets" [1]. In view of this, a study of the molecular mechanism of the action of various xenobiotics requires the development of methods that would permit registering the influence of substances on the membrane apparatus directly in an individual living cell. Promising in this respect is the fluorescent probe method, which is now being increasingly widely used in pharmacology [2]. Fluorescent probes react to a change in the permeability, surface charge of the membranes, transmembrane potentials, rearrangements of protein–lipid assemblies, etc. However, such high sensitivity of the probes to the most varied characteristics of the membranes also creates definite difficulties in the interpretation of the results in such a complex system as the living cell. The creation of a system of measurements in which information obtained with the aid of several mutually supplementing fluorescent probes is analyzed will permit these difficulties to be overcome.

In the present work we used a system of two oppositely charged probes: a membrane negatively charged probe of 1-anilinonaphthalene-8-sulfonate (1-N-phenylaminonaphthyl sulfonate, ANS), and a polychromatic positively charged probe, 4-(p-dimethylaminostyryl)-1-methylpyridine p-toluenesulfonate (DSM) [3], and various charged and uncharged xenobiotics of different classes.

EXPERIMENTAL

Fluorescent Probes. In the work we used ANS from Serva (Federal Republic of Germany) and DESM synthesized by G. Ya. Dubur and R. R. Dubur at the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR [4]. The spectral characteristics of the probes DSM and ANS are presented in [4, 5].

Production of Cells. The isolation of lymphocytes from the rat thymus, production of spermatozoa of the river loach and cells of protozoa of the species Tetrahymena pyriformis were described earlier [3]. A thymocyte suspension was prepared in Hanks' medium (Institute of Poliomyelitis and Viral Encephalitides, Moscow). The viability of the cells in the control experiments was 93-96% (test with trypan blue).

Staining of Living Cells. Solutions of DSM or ANS in the incubation medium were added to the suspension of living cells to final concentrations of 10 or 40 mM, respectively. Time of incubation 20 min.

Incubation of Cells with Xenobiotics. The following domestic chemical substances and pharmacological preparations were used: cetyltrimethylammonium bromide (I), dimedrol (II), aminazine (III), trifluoperazine (IV), amidopyrine (V), dioxidine (VI), actinomycin D (VII), and 2,4-dinitrophenol (VIII).

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