STRUCTURE CHARACTERIZATION OF REACTION PRODUCTS FROM PHENYLHYDRAZONOPROPANEDINITRILE AND THIOLS

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Received December 2nd, 1983

Phenylhydrazonopropanedinitriles react with thiols (benzyl mercaptan, cysteine, 2-mercaptoethanol, mercaptoacetic acid, glutathione, and alcohol dehydrogenase) to give addition products in which thiolate anion is attached to electrophilic carbon atom of cyano group. The reaction ability and way of reaction of phenylhydrazonopropanedinitriles are discussed in the context of mechanism of biological activity of these compounds, particularly that of their uncoupling effect on oxidative phosphorylation.

The phenylhydrazonopropanedinitrile derivatives substituted in the aromatic ring represent a group of bioactive compounds with a very broad spectrum of effects[1-7]. In the biochemical literature they are known, under the name carbonylcyanidephenylhydrazones, especially as very efficient agents for uncoupling of oxidative phosphorylation[8-10]. 4-Trifluoromethoxyphenylhydrazonopropanedinitrile and 3-chlorophenylhydrazonopropanedinitrile are used as standard uncouplers. Many times they proved to be valuable tools in studies of biological processes connected with energy transformations[3-13]. Also very important though less known is the fact (connected with the nature of their effect) that thiols can interfere with the uncoupling effect as well as with other biological effects[9-13]. Direct chemical modification of the SH groups involved in the oxidative phosphorylation process and/or other processes could be responsible for molecular mechanism of their action.

The reactions of phenylhydrazonopropanedinitriles with low-molecular thiols and SH enzymes under *in vitro* conditions were described[14,15], but the discrepancies concerning the site of the addition of thiol to the heterocumulene grouping initiated both a more detailed study of structure of the phenylhydrazonopropanedinitriles themselves[16,17] and a better characterization of their reaction products with thiols.

EXPERIMENTAL

The melting points were determined by means of a Kofler block and are not corrected. The UV spectra were measured in phosphate buffer (pH 7·05) with a Specord UV-VIS apparatus (Zeiss, GDR). The IR spectra were measured in KBr discs with a Perkin–Elmer 2002 (U.S.A.) and a Zeiss UR 20 (GDR) apparatus. The ^{13}C NMR spectra were measured with a JEOL FX-100 apparatus (Japan) at the working frequency of 25·047 MHz. The standard spectral width was 6002 Hz with the proton noise decoupling. The samples were prepared by dissolution in dimethyl sulphoxide (saturated solutions) and adjusting "pH" about 10. The mass spectra were measured with a MS902 S apparatus (A.E.I., Great Britain) with direct inlet system at the ionisation energy of electrons 70 eV and ionisation current of 100 μA. TLC (Silufol, Kavalier, ČSSR; benzene–ethyl acetate 5 : 3) was used to check the reaction course and purity of the substances.

Alcohol dehydrogenase from yeasts (Reanal, Hungary). Benzyl mercaptan was prepared according to Gemeiner and coworkers[18]. Phenylhydrazonopropanedinitrile (*I*) was synthesized according to Heytler[8], m.p. 147°C. For $C_9H_5N_4$ (169·2) calculated: 63·53% C, 3·53% H, 32·94% N; found: 63·21% C, 3·62% H, 33·17% N.

S-Benzyl (2-phenylhydrazono)cyanothioacetimidate (*II*)

Mixture of 0·5 g *I* and 1·4 ml benzyl mercaptan was refluxed in 20 ml methanol 5 h, the solvent was distilled off, and the residue was recrystallized from benzene–hexane mixture. Yield 78%, m.p. 121°C. For $C_{16}H_{14}N_4S$ (294·4) calculated: 63·31% C, 4·76% H, 19·05% N, 10·88% S; found: 65·30% C, 4·78% H, 18·70% N, 10·90% S.

Addition Product of *I* with Cysteine (*III*)

Solution of 0·3 g *I* in 4 ml methanol was mixed with solution of 3 g cysteine in 150 ml water, pH was adjusted at 7 (Na_2CO_3), and the mixture was stirred at 30°C 1 h. Then it was acidified to pH 3 (HCl), the precipitated solid was collected by suction, washed with water (5 × 50 ml), dried, and recrystallized from aqueous methanol. M.p. 130 – 131°C, yield 47%. For $C_{15}H_{14}N_4O_4 \cdot S_2$ (378·4) calculated: 47·82% C, 3·70% H, 14·81% N, 16·93% S; found: 47·90% C, 3·85% H, 15·01% N, 16·50% S.

Reaction Product of *I* with 2-Mercaptoethanol (*IV*)

Mixture of 0·3 g *I* and 1·5 g 2-mercaptoethanol in 4 ml methanol was neutralized with Na_2CO_3 (pH 7) and stirred at 30°C 1 h. Then it was acidified to pH 3 (HCl), and the precipitate obtained was washed with water–methanol 1 : 1 mixture (5 × 50 ml). The product was recrystallized, yield 72%, m.p. 212°C. For $C_9H_8N_4O$ (188·2) calculated: 57·45% C, 4·26% H, 8·51% N; found: 57·23% C, 4·48% H, 8·40% N. Mass spectrum *m/z* (%) 188 (M^+ corresponds to $C_9H_8N_4O$), 171 (7·9), 170 (26·3), 149 (39·5), 144 (7·9), 143 (26·3), 105 (18), 93 (10), 92 (18), 91 (26), 78 (12·5), 77 (100), 65 (17·1).

((2-Phenylhydrazono)-3-iminopropanenitrile)-S-thioacetic Acid (*V*)

Solution of 0·17 g *I* in 2 ml methanol was added gradually to 2·5 g sodium mercapto acetate in 30 ml water (pH 7·0; Na_2CO_3). The mixture was acidified to pH 3 (HCl) after 2 h, whereupon the isolated solid was washed with water (5 × 20 ml) and recrystallized from benzene–hexane mixture. M.p. 205°C (decomp.), yield 33%. For $C_{11}H_8N_4O_2S$ (260·3) calculated: 50·38% C, 3·82% H, 21·37% N, 12·21% S; found: 50·05% C, 3·96% H, 21·21% N, 11·68% S.

The compound *V* was also prepared in non-aqueous medium. Solutions of 0·34 g *I* in 100 ml benzene was treated with 2·62 g mercaptoacetic acid and stirred at 30°C 16 h, whereupon the solvent was evaporated, the residue was washed with 3 × 100 ml water (pH 3, HCl), and the product was recrystallized from benzene–hexane mixture. Yield 65%, m.p. 205°C. Mass spectrum *m/z* (H) 262 (M^+ is not present; the peak corresponds to $C_{11}H_{10}NH_4O_2S$), 244 (27), 105 (17) 93 (35), 92 (4·2), 77 (100), 65 (5·8), 51 (13).

N-Butyl-(2-phenylhydrazono)cyanacetamidine (*VI*)

Mixture of 0·3 g *I* in 10 ml methanol and 10 ml butylamine was refluxed 5 h, poured (while warm – 65°C) onto water, the precipitate was washed with 5 × 50 ml water (pH 3, HCl), and the product was recrystallized from aqueous methanol. Yield 85%, m.p. 135°C. For $C_{13}H_{16}N_5$ (202·3) calculated: 64·20% C, 7·00% H, 28·80% N; found: 64·33% C, 7·16% H, 28·48% N.

Reaction of *I* with Glutathione

Mixture of 500 mg glutathione and 30 ml water (pH 7, KOH) was stirred with 100 mg *I* in 2 ml methanol at room temperature 2 h and poured onto 150 ml acetone. The precipitate formed was filtered off and washed with 3 × 150 ml acetone. The product *VII* could not be purified to a satisfactory degree and was only analyzed by NMR technique.

Reaction of *I* with Alcohol Dehydrogenase (ADSH)

Lyophilized yeast alcohol dehydrogenase (1 g, *i.e.* $2 \cdot 10^{-4}$ mol SH groups) in 10 ml water (pH 7·0) was mixed with 0·1 g *I* in 2 ml methanol at room temperature, and the mixture was poured in 100 ml acetone. The precipitate formed was collected and washed to remove free *I* from the product *VIII*.

RESULTS

The reactions of phenylhydrazonopropanedinitriles with thiols and amines give products whose absorption maxima are shifted to higher wavelengths (Table I). This bathochromic shift indicates that the reaction is connected with an extension of electron conjugation in the reaction products as compared with the reactants.

The most significant IR spectral bands of phenylhydrazonopropanedinitriles have the absorption maxima about 2 200 cm^{-1}. These bands are due to valence vibrations of the nitrile groups. Due to asymmetry of the phenylhydrazonopropanedinitrile molecule and/or non-equivalence of the nitrile group caused by intermolecular hydrogen bonds[16,17], the IR spectrum exhibits two signals assigned to nitriles (Table I). The reaction products from phenylhydrazonopropanedinitrile and the nucleophilic reagents studied exhibited IR spectra from which at least one of the above-mentioned bands was absent (Table I). Therefrom it can be concluded that the thiols or amines react with nitrile group of phenylhydrazonopropanedinitrile.

The mass spectrum of compound *V* indicates that the substance is an 1 : 1 adduct, although the spectrum lacks the molecular ion. Analysis of the mass spectrum, however, cannot provide information on the position of attack of phenylhydrazono-

propanedinitrile by the thiols studied. The fragments found can be formed equally well by the attack of azomethine bond or of nitrile bond. The molecular ion m/z (188) of compound *IV* indicates that the reaction product contains a water molecule added to the heterocumulene grouping, which is obviously a consequence of hydrolysis of the primary addition product. As in the previous case it is impossible to draw conclusions about the position of attack from the mass spectral analysis. Therefore, we did not characterize the mass spectra of the reaction products of phenylhydrazonopropanedinitrile with glutathione or butylamine either. The reaction product with benzyl mercaptan could not be analyzed by mass spectrometry due to its tendency to decompose into the original components. In case of the reaction product with cysteine the mass spectrum exhibits ions with m/z values above 292, which could be explained by at least two molecules of cysteine participating in the reaction.

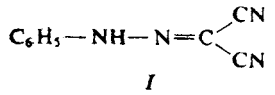
The chemical shifts of carbon atoms of the aromatic nucleus depend strongly on nature of the structural fragment bound to the nitrile group. Such variation of chemical shifts of aromatic derivatives with substituents bound by means of a nitrogen atom is possible[19] and is connected obviously with steric effects on transmission of polar effects of the substituent through the hydrazone bridge of the molecule. Therefore, the individual signals of the ^{13}C NMR spectrum were interpreted predominantly on the basis of their chemical shifts. Comparison of the resonance spectra of the starting compound *I* and the products *II*–*VI* (Table II) shows that the reac-

TABLE I

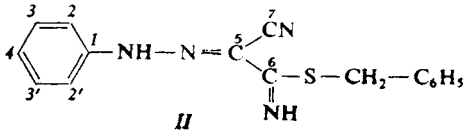
Spectral parameters of the products *I*–*VIII*

Compound	IR region					UV and VIS λ_{max}
	ν(C≡N)	ν(C≡N)	ν(C=N)	ν(C=O)	ν(NH)	
I	2 215	2 239	1 604	—	3 280	376
II	2 218	—	1 600	—	3 295	401
III	—	—	1 599	1 740	3 070	392
IV	2 310	—	1 615	1 708	3 300	365
V	2 210	—	1 580	1 717	3 150	416
VI	2 182	—	1 635	—	3 299 3 410	396
VII	2 217	—	1 610	1 740	3 200	395
VIII	2 235	—	1 570	1 780	3 300	395

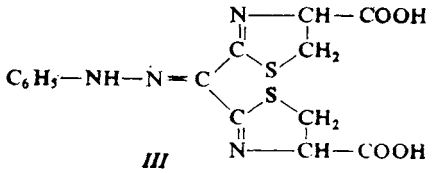
tion did not take place in the phenyl ring. However, the existence of the signal at $\delta \sim 160-165$ ppm in spectra of the reaction products unambiguously confirms that the reaction did not take place at the azomethine bond —N=C< either, the reaction centre being the nitrile groups. In most cases one nitrile group only reacted. This fact was manifested in the ^{13}C NMR spectrum by an increase in the chemical shift of azomethine group by as much as 20 ppm and by formation of a signal of imino group >C=NH (about 160 ppm). Cysteine is added to the both nitrile groups, but with regard to asymmetry of the molecule[16,17] the cysteine residues are not equivalent, which results in doubling of the respective NMR signals. Disappearance of the second nitrile group is manifested by further increase of the chemical



I

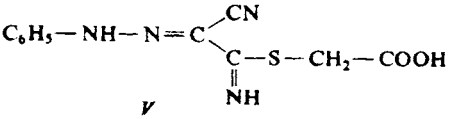


II

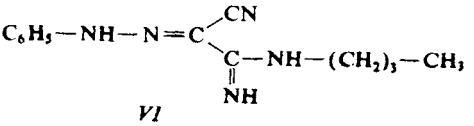


III

IV



V

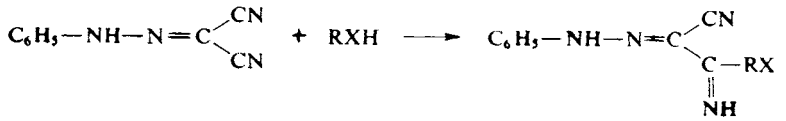


VI

shift of the carbon atom in the azomethine grouping (Table II). It is substantially more difficult to interpret the spectrum of the reaction product of phenylhydrazonopropanedinitrile with glutathione. The spectrum is much too complicated, the substance being a mixture of 3–4 compounds. It is, nevertheless, evident from the spectrum that the reaction represents addition to nitrile group in this case, too.

DISCUSSION

Structural analysis of the reaction products of phenylhydrazonopropanedinitrile with the model nucleophilic reagents unambiguously shows that both thiols and amines react with the carbon atom of the nitrile group according to the equation (*A*).



(*A*)

In case of reagents containing one nucleophilic group only, the addition reaction can only proceed at one nitrile group at physiological conditions (the cases *II*, *V*, *VI*, *VII*). Obviously, at more drastic conditions, the addition to the other cyano group can take place, too[14]. With bifunctional nucleophiles other reactions (predominantly

TABLE II

The ^{13}C NMR chemical shifts of compounds *I*–*VI* in δ scale; for numbering of atoms see formula *II*

Compound	1	2,2′	3,3′	4	5	6	7	The other carbon atoms
I	142·1	117·1	130·5	126·8	86·5	114·2	109·8	—
II[a]	152·5	121·7	129·8[b]	128·8[b]	106·1	165·0	115·4	CH_2— 34·4
III	142·0	115·1	129·8	124·8	124·6	163·8	163·8	CH 78·9; CH_2 33·3; COOH — 171·9 75·1 32·8
IV	142·2	115·8	129·1	123·9	107·7	162·9	115·5	—
V	153·0	120·9	129·3	127·5	104·8	162·4	115·2	CH_2— 36·4; COOH— 187·7
VI	153·6	120·3	128·5	125·0	91·0	159·3	116·0	NCH_2CH_2— 30·9; NCH_2— 41·0 CH_3— 13·7; CH_3CH_2— 19·4

[a] The chemical shifts of phenyl ring in benzyl mercaptan: C_{ipso} — 136·0; C_{ortho} — 129·9[b]; C_{meta} — 129·7[b]; C_{para} — 128·7[b]. [b] The assignment of chemical shifts is not unambiguous.

eliminations) can also be significant. In the case of 2-mercaptoethanol, the primary adduct undergoes further transformations. Cysteine is added to the both nitrile groups, the structure *III* being consistent with the analyses carried out. The reactivity of amino group in butylamine as a model amino compound is negligible in comparison with that of sulfhydryl group of the selected thiols (benzyl mercaptan, mercaptoacetic acid) in aqueous medium. Thus an incubation of phenyhydrazonopropanedinitrile with butylamine (2 h, pH 7, 25°C) caused no change in composition of the mixture, the corresponding reaction with thiols at comparable conditions being quantitative. This fact allows application of phenylhydrazonopropanedinitrile as a selective SH reagent in biochemistry. It is also decisive from the point of view of mechanistic studies of biological activity of phenylhydrazonopropanedinitriles: during interactions with proteins at physiological conditions these dinitriles will highly prefer the reactions with SH groups. On the other hand, chemical modification of other nucleophilic functional groups (—NH_2, —OH) in proteins is considerably improbable. These findings can be important especially for understanding the molecular mechanism of ATP synthesis in the course of oxidative and photosynthetic phosphorylations. There exist different ideas about participation of thiol and other functional groups of proteins in this process[20–23]. Thus phenylhydrazonopropanedinitriles, as efficient uncouplers of oxidative phosphorylation and selective SH reagents, can represent a useful tool for studies of intimate chemical mechanisms of energy formation and transformations in biological systems. The findings about the ability of phenylhydrazonopropanedinitriles to bind glutathione (a model thiol tripeptide with one SH group) or alcohol dehydrogenase (M.w. 150 000, with 32 to 36 SH groups) were utilized in preparation of polymeric phenylhydrazonopropanedinitriles for use as sorbents of biological thiol compounds[24].

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Translated by J. Panchartek.