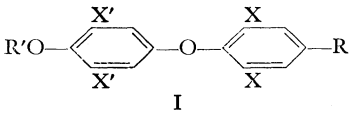
9. This analysis was carried out in the laboratory of Dr. Karl Meyer, Department of Medicine, College of Physicians and Surgeons, Columbia University. The methods have been described by K. Meyer *et al.* [*Biochem. et Biophys. Acta* 21, 506 (1956)] and by P. J. Stoffyn and R. W. Jeanloz [*Arch. Biochem. Biophys.* 52, 373 (1954)].

27 November 1957

Sterically Hindered Analogs of Thyroxine

In previous papers from this laboratory (*1–4*) the synthesis of various compounds of general structure I, related to thyroxine, was reported. In structure I, R = $-CH_2CH_2COOH$, $-CH_2CH(NH_2)COOH$, $-NH_2$, etc; X = iodine; X′ = iodine, methyl, etc.; and R′ = methyl or hydrogen.

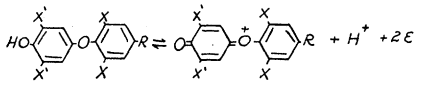


I

In an accompanying paper (*5*), an empirical correlation between structure and biological activity for 47 analogs of structure I was proposed.

The above correlation, while entirely empirical in nature, suggested significant deductions about the essential pharmacogen which is required for thyroxine-like activity. It also led to the conclusions that structural parameters, such as the electron-releasing abilities of X, X′ and OR′, the hydrogen bonding abilities of X and X′ and the *pK* values for the compounds (dependent on the nature of the ionizing side chain, R), are the probable factors which determine the comparative biological activity of these substances. Of major importance to our thinking in arriving at the correlative conclusions was the very striking fact (*5*) that the 3′,5′-dimethyl analogs of L- and D,L- thyroxine (structure I, X = iodine, X′ = methyl, R′ = H) were distinctly more active (*5*), in certain assays of thyroxine-like activity, than the corresponding stereoisomers of thyroxine. The suggestion that substitution of electron-releasing groups, such as methyl, in place of electron-attracting groups such as iodine, bromine, nitro, and so forth, can enhance thyroxine-like activity is a novel one and is in direct opposition to earlier considerations pertaining to such effects (*5, 6*).

The postulate of the Bruice-Kharasch-Winzler correlation, that electron-releasing groups in the 3′,5′-positions of structure I can enhance thyroxine-like activity, finds a possible rationale in the hypothesis of Niemann (*7*) that oxidation of thyroxine to a quinoid form, as shown below, may somehow be involved in its action. Essentially, this oxidation



appears to involve removal of the elements of a hydride group ($H^+ + 2e$) from the thyroxine analog, and this reaction should be enhanced by substituting electron-releasing groups into the back ring of thyroxine.

The above predictions of activities, and the possible relations to the Niemann hypothesis, can be tested by synthesis and biological evaluation of suitable compounds (such as L- and D,L- 3,5,3′-triiodo-5′-methylthyronines and others) in which electron-releasing groups are incorporated into the structures related to compound I. Compare, for example, reference *3*, for the synthesis of initial substances for these purposes.

We now also wish to report our studies toward the synthesis of compounds related to structure I, in which the X′ groups have favorable electron-releasing abilities, but the steric characteristics of which should be such as to cause significant steric complications toward an in vivo oxidative reaction, which may be involved in converting the analog of I to a quinoid form, as illustrated in the equation above. The first compound of this type which we wish to report is the 3′,5′-di-tertiarybutyl analog (X′ = *t*-butyl) and with R = $-CH_2CH_2COOH$. This compound was synthesized by the route shown in Fig. 1.

Compound III melted at 94° to 95°C and gave the following analysis: Calcd. for $C_{25}H_{32}N_2O_8$: C, 61.48; H, 6.56; N, 5.74; found: C, 61.68; H, 6.66; N, 5.57. The conversion of III to I (X = iodine; X′ = *t*-butyl; R′ = H; R = $-CH_2CH_2COOH$) was carried out by reducing to the diamine, diazotization and use of the Sandmeyer reaction to introduce iodine at the 3,5-positions, and hydrolysis of the intermediate ester; m.p. 120° to 121°C. Analysis (for the ester): Calcd. for $C_{25}H_{32}O_4I_2$: C, 46.16; H, 4.96; I, 39.03; found: C, 46.55; H, 4.97; I, 39.29. The final product, as the free acid, was obtained as excellent colorless needles, from aqueous ethanol, which melted at 197° to 198°C. Analysis: Calcd. for $C_{23}H_{28}O_4I_2$: C, 44.39; H, 4.53; I, 40.79; found: C, 44.53; H, 4.60; I, 40.60.

In the above compound, if the *t*-butyl groups are sufficiently large to block the in vivo oxidative reaction to the quinoid form, and if they are not removable in vivo (a question of general interest, which has yet to be resolved), then the possibility of finding a true competitive inhibitor to thyroxine is implied in this approach. The synthesis of various such molecules (the so-called "hinderins") is therefore a major objective of our studies (*8*).

A sample of "hinderin A" (structure I: X′ = *t*-butyl; X = iodine; R′ = H; R = $-CH_2CH_2COOH$) has been submitted for biological assay (effect on metabolism of glucose in *Aerobacter* aerogenes) by W. Marx and M. Gutenstein, who have reported interesting results for the initial screening.

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8. This work was supported by the U.S. Public Health Service (grant No. A-703 (C3) and by the Travenol Laboratories of Morton Grove, Ill.

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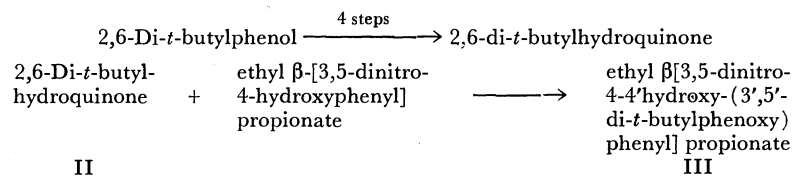

Fig. 1. Synthesis of the 3′,5′-di-tertiarybutyl analog of structure I.

On the Composition of Zymosan

Zymosan is the name given by Pillemer and Ecker (*1*) to a yeast fraction having the specific immunological property of inactivating the third component of complement, C′3. Later work indicated that zymosan adsorbs properdin, a radiation-sensitive serum protein reported to protect mammals from the spread of microbiological infection (*2*). Further interest in zymosan is derived from the finding that its injection into mice, rats, and rabbits results in an immediate decrease in the properdin titer followed by an increase to levels sometimes three times greater than the initial properdin level (*3*). Consistent with this property are the observations that zymosan decreases the lethal effect of x-radia-

tion (*4*) and increases the bactericidal activity of animal sera (*5*).

Zymosan preparations defined as types A and B on the basis of immunological tests were isolated from Fleischmann yeast by the procedure published by Pillemer *et al.* (*6*). Table 1 contains analytical data developed on three preparations of zymosan type A and on one sample of zymosan type B. The analytical differences between the two types of zymosan are no greater than the variations noted among different preparations of zymosan type A. Therefore, the difference in immunological activity of zymosan types A and B cannot be ascribed to gross chemical composition. One possibility is that surface structure and charges determine zymosan activity. Following is a brief description of the analytical procedures employed in this work.

Table 1. Composition of zymosan.

Component	Percentage by weight, dry basis			
	Type A			Type B
	7B13	7B152	7B340	6B506
Total polysaccharide	75.7	70.7	73.0	74.7
Glucan	57.8	53.8	50.7	56.7
Mannan	17.9	16.9	22.3	18.0
Total nitrogen	2.32	2.79	2.19	2.27
Protein nitrogen	2.26	2.73	2.13	2.20
Glucosamine nitrogen	0.06	0.06	0.06	0.07
Protein	14.1	17.1	13.3	13.8
Glucosamine	0.71	0.75	0.74	0.87
Chitin*	0.78	0.83	0.82	0.95
Fat	6.4	6.9	6.9	5.5
Ash	3.42	2.98	3.42	2.94
Phosphorus	0.79	0.77	0.79	0.71
Magnesium	0.28	0.28	0.28	0.51

* Calculated from the glucosamine analysis.

A zymosan sample (about 200 mg, accurately weighed) was hydrolyzed by shaking at 30°C with 10 ml of 22*N* sulfuric acid solution for 16 hours. The mixture was diluted with 40 ml of water and boiled under reflux for 1 hour. An insoluble material separated on cooling and was removed by filtration through sintered glass. The residue was washed thoroughly with water. The combined washings and filtrate were brought to *p*H 8 with 10*N* sodium hydroxide solution. The solution was concentrated by distillation under high vacuum and brought to a volume of 50 ml. [A total carbohydrate analysis performed with anthrone (*7*) showed that all of the polysaccharide present in zymosan was solubilized.] Aliquots were submitted to paper chromatography on 5½- by 17-inch sheets of Whatman No. 1 paper; the developing solvent was N-butyl alcohol, absolute ethyl alcohol, and water [2:1:1]. The multiple development technique (*8*) was employed over a period of 7 days—that is, the chromatograms were removed daily, dried, and returned to the developing tanks during the seven days that the chromatography was performed. Finally, the chromatograms were treated by conventional techniques for the identification of monosaccharides. Only glucose and mannose were present; analyses were conducted on eluates by the anthrone procedure (*7*).

Glucosamine analyses were conducted on the same sulfuric acid digest as that used for glucose and mannose assays. The modified method (*9*) of Elson and Morgan (*10*) was employed. Glucosamine was identified by previous work with zymosan hydrolyzates produced in sealed tube reactions with 2*N* hydrochloric acid at 100°C (*11*); this type of hydrolyzate was not used for quantitative work because carbohydrates other than simple sugars were present on the paper chromatograms. The identification of glucosamine in such hydrolyzates was based upon (i) the correspondence of its R_G with the value obtained from work with pure glucosamine, (ii) the positive response of the material to staining with aniline phthalate and with ninhydrin, (iii) the finding that the eluate produced a red solution with maximum absorption at 530 mμ on treatment with acetylacetone and *p*-dimethylaminobenzaldehyde (*12*), and (iv) the failure of the eluate to produce color with anthrone (*7*). A positive qualitative test for chitin was obtained by hydrolyzing zymosan with alkali in a sealed tube and producing a red-violet color by treating the resulting chitosan with potassium iodide–iodine solution (*13*). Zymosan samples were assayed for ash by dry combustion at 550°C to constant weight. Procedures described in the literature were used to determine fat (*14*), phosphorus (*15*), and magnesium (*16*). The ultraviolet absorption spectrum of zymosan hydrolyzates did not reveal the presence of nucleic acid split-products (*17*). On the average, the analytical values presented in Table 1 account for 98.6 percent of the material as 54.7 percent glucan, 18.8 percent mannan, 14.5 percent protein, 6.6 percent fat, 3.2 percent inorganic material, and 0.8 percent chitin. Zymosan contains both the yeast cell wall polysaccharides described by Northcote and Horne (*11*)—namely, the outer glucan shell and the inner mannan layer. It is interesting to note the ratio of mannan to glucan in the following materials: 1.6 for whole yeast (*18*); 1.0 for "cell-wall" (*11*), and 0.34 for zymosan (Table 1).

Recently, Falcone and Nickerson (*19*) prepared and analyzed yeast cell wall substance; their material, like zymosan, contains no nucleic acid. Zymosan contains twice as much nitrogen (2.36 percent) as yeast "cell wall" isolated by Falcone and Nickerson, and somewhat more nitrogen than the yeast "cell wall" preparation of Northcote and Horne (2.1 percent nitrogen).

Photomicrography confirms the statement that zymosan is derived from the yeast cell wall (*1*). Photomicrographs of Gram-stained zymosan and yeast show that zymosan consists of "ghost" cells. The average particle diameter of zymosan is about 3 μ; yeast cells have an average diameter of approximately 6 μ (*20*).

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