ACTA CHEMICA SCANDINAVICA 7 (1953) 806–812

# Synthetic Inhibitors of Hyaluronidase

## II. New Polycondensed Diphenylmethane and Triphenylmethane Derivatives

L. HAHN and E. FRANK

*Research Laboratory of AB Ferrosan, Malmö, Sweden*

In the preceding paper [1] we described the hyaluronidase inhibiting activity of some diphenylmethane derivatives obtained by condensation of hydroxy benzoic acids with formaldehyde. Analogous triphenylmethane derivatives were found to be even more active.

In a search for still more active inhibitors we studied the reaction between hydroxy benzoic acids and formaldehyde under varying conditions to find out any relation between the conditions under which the condensation was carried out and the inhibitory effect of the products obtained. It was found that the inhibitory effect of the condensation products varies with the ratio between the amounts of hydroxy benzoic acid and formaldehyde entering the reaction. With an excess of formaldehyde very active compounds of high molecular weight were obtained.

From the diphenylmethane derivatives thus prepared triphenylmethane derivatives were obtained by condensation with a phenol or a hydroxy benzoic acid under the influence of nitrous acid. Some triphenylmethane derivatives were also prepared in a similar way from condensation products of phenols with formaldehyde.

The purpose of this paper is to report an investigation of the inhibitory effect of these new substances on hyaluronidase *in vitro* and their oral toxicity in mice.

### METHOD

*Substrate solution.* 2 g sodium hyaluronate prepared from human umbilical cord by the method of Blix [2] were dissolved in 1 litre $M/6.5$ phosphate buffer solution of pH 7. The solution thus prepared was stored in the refrigerator.

*Enzyme solution.* Unless otherwise stated, use was made of hyaluronidase extracted from the bull's testes and purified by the method of Hahn [3]. The stock solution was kept in the refrigerator, and dilutions were prepared with isotonic saline once a week for laboratory use.

*Preparation of the test substances.* The substances to be tested for inhibition of hyaluronidase activity were dissolved in diluted sodium hydroxide solution, and the pH was adjusted to 7. The solutions were made isotonic by the addition of saline.

*Method of determination of inhibitory power.* The estimations were made by the method described in the preceding paper. In the present assays a product obtained by the condensation of gentisic acid with formaldehyde (compound 7) was used as standard. The relative inhibitory power of this compound was 1 200 (resorcinol = 1) *.

*Toxicity* **. The test substance was emulsified in an aqueous solution of gum arabic and sugar. The concentration of the emulsion varied from 2 per cent to 30 per cent according to the size of the dose used. Single doses of 0.01—0.05 ml of the emulsion per g bodyweight were given by stomach tube to female mice weighing 20—24 g. Six dosage levels of every substance were studied. Each dosage level was tested on 6 mice. The animals were then closely observed for one week. When death occurred it usually happened within 48 hours. The $LD_{50}$ values were determined by the Behrens method, as modified by Kärber [4] and expressed in mg/g.

## RESULTS

As mentioned in the preceding paper, the inhibitor built up of gentisic acid units was the most powerful of the condensation products of diphenylmethane type studied. Therefore, when studying the effect that modifications of the conditions under which hydroxy benzoic acids are condensed with formaldehyde might exert on the inhibitory power of the resultant product, gentisic acid was chosen as a test substance. Observations made in these trials showed that the inhibitory power of the condensation products within certain limits varies with the ratio between the amount of gentisic acid and formaldehyde entering the reaction. Thus, when 4 parts of gentisic acid were allowed to react on the boiling water bath for five hours with 1 part of 40 per cent formaldehyde (molecular ratio 1 : 0.5) in the presence of 20 parts of 50 per cent (v/v) sulfuric acid, the inhibitory power of the substance obtained was 1 200 rel. units, while the corresponding value of a product prepared with twice the amount of formaldehyde but under otherwise identical conditions was 2 000. This last-mentioned condensation product (compound 7 P, "Digentisic acid") consisted mainly of compounds of high molecular weight, as demonstrated by dialysis of a solution of its sodium salt through cellophane at pH 7, when only about 15 per cent passed through the membrane. A further increase of the amount of formaldehyde was found to have no appreciable effect on the activity of the product.

---

* *Cf* Table 2 in the preceding article.

** The toxicity tests were carried out by Dr. M. Fabinyi-Szebehely.

*Table 1. Hyaluronidase-inhibiting power of polycondensed diphenylmethane derivatives*

| Compound condensed with formaldehyde in the molecular proportion of 1 : 1 | Condensation product Code name | No. | Hyaluronidase inhibiting power in relative units (resorcinol = 1)* | Acute oral toxicity in mice $LD_{50}$ |
|---|---|---|---|---|
| Salicylic acid | "Disalicylic acid" | 23 | 220 (50) | 4.8 |
| *p*-Hydroxy-benzoic acid | "Di-*p*-hydroxy benzoic acid" | 2 P | 400 (380) | 4.5 |
| Gentisic acid | "Digentisic acid" | 7 P | 2 000 (1 200) | 10.0 |
| Protocatechuic acid | | 22 | 1 120 | 10.0 |
| *α*-Resorcylic acid | | 8 P | 800 (760) | |
| *β*-Resorcylic acid | "Di-*β*-resorcylic acid" | 9 P | 560 (400) | |
| *γ*-Resorcylic acid | "Di-*γ*-resorcylic acid" | 19 P | 1 700 (980) | 4.6 |
| Gallic acid | "Digallic acid" | 1 P | 350 (250) | |
| 2,3,4-Trihydroxy benzoic acid | | 63 | 510 | |
| 2,4,6-Trihydroxy benzoic acid | | 3 P | 900 (780) | |
| 2,4,5-Trihydroxy benzoic acid | | 58 | 620 | |
| Vanillic acid | | 24 | 360 | |
| Hydroquinone | "Dihydroquinone" | 27 | — | |
| Pyrogallol | "Dipyrogallol" | 25 | — | |
| Resorcinol | "Diresorcinol" | 26 | — | |
| Phloroglucinol | "Diphloroglucinol" | 12 P | — | |

* Bracketed figures denote the inhibitory power of the corresponding product obtained on condensation with formaldehyde in the molecular ratio of 1 : 0.5.

These results prompted us to condense other hydroxy benzoic acids with formaldehyde in the molecular proportion of 1 : 1 under conditions otherwise identical with those described in the preceding article. The products thus obtained consisted to a great part of high molecular compounds. The condensation product of *β*-resorcylic acid and formaldehyde (compound 9 P), for example, showed a content of 79 per cent non-dialyzable substance and the corresponding figure for the condensation product of *p*-hydroxy benzoic acid (compound 2 P) was found to be 63 per cent. As a rule the inhibitory powers of these products were appreaciably higher than those of the products prepared by condensation of the corresponding hydroxy benzoic acid with formaldehyde in a molecular ratio of 1 : 0.5 (Table 1). Condensation in proportions $1 : > 1$ did not yield products of still higher activity. Some phenols were also condensed with formaldehyde in a molecular proportion of 1 : 1. None of the products thus obtained were soluble enough to permit any estimation of their inhibitory power *in vitro*.

Polycondensed products built up of triphenylmethane units were prepared by oxidation of the above-mentioned polycondensed diphenylmethane derivatives in the presence of a phenol or a hydroxy benzoic acid with nitrous acid. Use was made of the method described in the preceding article. The inhibitory powers of all of the substances tested are summarized in Table 2. Many of these compounds possess a higher inhibitory power than any substance hitherto described. The most active product was compound 21 P ("Trigentisic acid").

The triphenylmethane derivatives listed in Table 2 differ from one another by the number and position of the hydroxy and carboxy groups and presumably by the degree of condensation. This applies to the diphenylmethane derivatives, too (Table 1).

As yet the exact chemical formulas of these condensation products of diphenylmethane and triphenylmethane type cannot be given. The first-mentioned products may be conceived as long chain molecules built up of substituted benzene rings linked together by methylene groups. Except for the condensation products of trihydroxy benzoic acids these chains may also be branched. As to the structure of the triphenylmethane derivatives described here, it must be borne in mind that when building up triphenylmethane molecules by the introduction of a radical into the methylene bridge of diphenylmethane molecules, the nuclei of the diphenylmethane molecules themselves may compete with the phenol or carboxyphenol molecules.

Observations hitherto made in the present material do not allow of any definite conclusions regarding the possible relationship between the number and position of the substituents in the aromatic nuclei of the condensation products and the inhibitory activity of these substances. The most active diphenylmethane derivatives are the condensation products of dihydroxy benzoic acids. The most powerful condensation products of triphenylmethane type have 5—7 hydroxy groups per triphenylmethane unit. This suggests that the number of hydroxy groups is of importance for the inhibitory power of the compounds. Furthermore, the diphenylmethane and triphenylmethane derivatives built up of salicylic acid possessed but weak inhibitory power and were much less active than the corresponding derivatives of *p*-hydroxy benzoic acid. This indicates that the inhibitory power of the products may vary with the position of the hydroxy groups. This suggestion is supported by the difference observed between the inhibitory power of corresponding condensation products of different dihydroxy benzoic acids.

Work is in progress at this laboratory [5] to clear up the question whether the degree of condensation influences the inhibitory power of the condensation products described here. Suffice it here to mention that an increase in the quantity of formaldehyde in the condensation of phenols or hydroxy benzoic

*Table 2. Hyaluronidase-inhibiting power of triphenylmethane derivatives obtained on reaction of a polycondensed diphenylmethane derivative with a phenol derivative in the presence of an oxidizing agent.*

| Diphenylmethane derivative | Phenol derivative | Condensation product No. | Hyaluronidase inhibiting power in relative units (resorcinol = 1) | Acute oral toxicity in mice $LD_{50}$ |
|---|---|---|---|---|
| "Disalicylic acid" | Salicylic acid | 35 | < 200 | |
| "Di-*p*-hydroxy-benzoic acid" | *p*-Hydroxy benzoic acid | 20 P* | 1 000 | 2.7 |
| "Di-*p*-hydroxy-benzoic acid" | Gentisic acid | 36 | 1 550 | 6.3 |
| "Di-*p*-hydroxy-benzoic acid" | β-Resorcylic acid | 38 | 1 300 | 3.8 |
| "Di-*p*-hydroxy-benzoic acid" | Gallic acid | 37 | 2 000 | 5.6 |
| "Di-*p*-hydroxy-benzoic acid" | Hydroquinone | 39 | 1 100 | 9.0 |
| "Di-*p*-hydroxy-benzoic acid" | Phloroglucinol | 40 | 2 100 | 18.0 |
| "Digentisic acid" | Salicylic acid | 28 | 2 100 | 8.7 |
| "Digentisic acid" | *p*-Hydroxy benzoic acid | 29 | 1 950 | > 20.0 |
| "Digentisic acid" | Gentisic acid | 21 P** | 2 500 | > 20.0 |
| "Digentisic acid" | β-Resorcylic acid | 31 | 2 250 | 6.5 |
| "Digentisic acid" | Gallic acid | 30 *** | 2 200 | 12.5 |
| "Digentisic acid" | Resorcinol | 32 | 1 650 | > 20.0 |
| "Digentisic acid" | Hydroquinone | 33 | 1 900 | 18.0 |
| "Digentisic acid" | Phloroglucinol | 34 | 1 600 | 19.5 |
| "Di-β-resorcylic acid" | *p*-Hydroxy benzoic acid | 42 | 2 000 | 4.6 |
| "Di-β-resorcylic acid" | Gentisic acid | 41 | 1 900 | 4.4 |
| "Di-β-resorcylic acid" | β-Resorcylic acid | 43 | 1 300 | 3.5 |
| "Di-β-resorcylic acid" | Gallic acid | 16 P **** | 1 300 | 5.4 |
| "Di-β-resorcylic acid" | Hydroquinone | 45 | 2 300 | 4.5 |
| "Di-β-resorcylic acid" | Phloroglucinol | 44 | 2 200 | 5.0 |
| "Di-γ-resorcylic acid" | Gentisic acid | 59 | 2 350 | 3.8 |
| "Di-γ-resorcylic acid" | γ-Resorcylic acid | 60 | 1 900 | 4.0 |
| "Di-γ-resorcylic acid" | Hydroquinone | 61 | 1 800 | |
| "Di-γ-resorcylic acid" | Phloroglucinol | 62 | 2 300 | 5.0 |
| "Dipyrogallol" | Salicylic acid | 53 | 1 350 | |
| "Dipyrogallol" | *p*-Hydroxy benzoic acid | 54 | 650 | |
| "Dipyrogallol" | Gentisic acid | 51 | 700 | |
| "Dipyrogallol" | β-Resorcylic acid | 52 | 1 700 | 18.5 |
| "Dipyrogallol" | Gallic acid | 50 | 2 000 | 17.5 |
| "Diresorcinol" | Gallic acid | 47 | 300 | |
| "Dihydroquinone" | Salicylic acid | 55 | 300 | |
| "Dihydroquinone" | Gentisic acid | 56 | 250 | |
| "Dihydroquinone" | β-Resorcylic acid | 57 | 480 | |
| "Diphloroglucinol" | *p*-Hydroxy benzoic acid | 17 P | 700 | |
| "Diphloroglucinol" | Gentisic acid | 48 | 750 | |
| "Diphloroglucinol" | β-Resorcylic acid | 49 | 1 650 | 6.0 |
| "Digallic acid" | β-Resorcylic acid | 46 | 100 | |

* "Tri-parahydroxy-benzoic acid"
** "Trigentisic acid"
*** "Digentisic-gallic acid"
**** "Di-β-resorcylic-gallic acid"

acids, which appears to favour the formation of products of high molecular weight, resulted in the formation of more active diphenylmethane and triphenylmethane derivatives. In this connection it should perhaps be pointed out that the number and position of hydroxy groups may influence the mechanism of the condensation and thereby also the degree of polycondensation.

*Inhibition of staphylococcal hyaluronidase.* Whether the compounds described above inhibit hyaluronidase derived from sources other than mammalian testes is a question of great importance in the investigation of the mechanism by which hyaluronidase of varying origin breaks down hyaluronic acid and for defining the range of indications for these inhibitors in the clinic. The inhibitory action of some of the compounds here described on hyaluronidase obtained from different strains of pathogenic bacteria will be the subject of a later paper. It may be sufficient here to mention that hyaluronidase obtained from a strain of *Staphylococcus pyogenes* was inhibited to roughly the same extent as testis hyaluronidase. For example, compound 21 P was investigated in a concentration which in our test with testis hyaluronidase gives a fivefold prolongation of the half-viscosity time. Using staphylococcus hyaluronidase under strictly identical conditions a 3.5 fold prolongation was obtained. The corresponding figures for compound 7 P and 20 P were 3.6 and 2.1 respectively.

*Toxicity.* The products with the most pronounced inhibitory power were tested for acute oral toxicity in mice (Tables 1 and 2). 8 of the compounds showed $LD_{50}$ values above 15 mg/g, compound 21 P and two related compounds being the least toxic. The remarkably low toxicity of some of these substances is important to the possible clinical value of the hyaluronidase inhibitors mentioned in the preceding paper. A more detailed study of the toxicity of compound 21 P and some other compounds as well as of their inhibitory action on hyaluronidase *in vivo* is the subject of another paper [6]. The results of a clinical trial with compounds 7 P, 16 P and 21 P in rheumatoid arthritis have been published elsewhere [7].

## SUMMARY

A further study was made on the inhibition of testis hyaluronidase by diphenylmethane and triphenylmethane derivatives with hydroxy and carboxy groups in the nuclei.

A number of mono- and polyhydroxy benzoic acids were condensed with formaldehyde in the molecular ratio of 1 : 1. Dialysis of the diphenylmethane derivatives obtained showed that they mainly consist of high molecular polycondensation products. They possessed higher inhibitory power than the corresponding products obtained by condensation in the molecular ratio of

1 : 0.5 as described earlier. The reaction product of gentisic acid with formaldehyde, compound 7 P ("Digentisic acid"), was found to be most powerful (relative inhibitory activity: 2 000; resorcinol = 1).

Polycondensation products built up of triphenylmethane units were produced by the oxidation of polycondensed diphenylmethane derivatives with nitrous acid in the presence of a phenol or a hydroxy benzoic acid. Altogether 38 substances of triphenylmethane type were synthesized and examined for their inhibitory action on hyaluronidase *in vitro*. Of these, 9 possessed an inhibitory power of more than 2 000 rel. units. Compound 21 P ("Trigentisic acid"), a product obtained by oxidizing compound 7 P in the presence of gentisic acid, proved most active with an activity of 2 500 relative units.

Compounds 21 P, 7 P and 20 P ("Tri-parahydroxy benzoic acid", a *p*-hydroxy benzoic acid analogous to compound 21 P) were also tested for inhibitory effect on hyaluronidase from a strain of *Staphylococcus pyogenes*. The inhibitory action found was roughly the same as on testis hyaluronidase.

The acute oral toxicity in mice of the most active polycondensed diphenylmethane and triphenylmethane derivatives was assessed. Compound 21 P and two closely related derivatives, obtained by the oxidation of compound 7 P in the presence of *p*-hydroxy benzoic acid and resorcinol respectively, proved least toxic ($LD_{50} > 20$ mg/g body weight). The $LD_{50}$ values of further 5 triphenylmethane derivatives exceeded 15 mg/g.

Thanks are due to Mr. G. Söderberg, E. Eriksson and A. Anderberg for valuable technical assistance.

## REFERENCES

1. Hahn, L., and Fekete, J. *Acta Chem. Scand.* **7** (1953) 798.
2. Blix, G., and Snellman, O. *Arkiv Kemi, Mineral. Geol.* **19 A** (1945) No. 32.
3. Hahn, L. *Biochem. Z.* **315** (1943) 83.
4. Kärber, G. *Arch. exptl. Pathol. Pharmakol.* **140** (1929) 237.
5. Ekström, G., and Hahn, L. *unpublished data.*
6. Fabinyi-Szebehely, M., Hahn, L., and Szebehely, *J. Brit. J. Pharmacol.* **8** (1953) 30.
7. Hahn, L., Thune, S., and Truedsson, E. *Ann. Rheumatic Diseases* **11** (1952) 272.

Received February 11, 1953.