Biochemical Pharmacology, Vol. 19, pp. 2501–2507. Pergamon Press. 1970. Printed in Great Britain

EFFECT OF ESTER GROUPS ON THE HAEMOLYTIC ACTION OF SAPOGENINS—II

ESTERIFICATION WITH BIFUNCTIONAL ACIDS

RUTH SEGAL, ILANA MILO-GOLDZWEIG, H. SCHUPPER and D. V. ZAITSCHEK

Department of Pharmacognosy, School of Pharmacy,
Hebrew University, Jerusalem, Israel

(*Received* 2 *February* 1970; *accepted* 25 *March* 1970)

Abstract—The cause for the enhancement of haemolysing capacity of sapogenin esters was investigated. For this purpose the haemolysis induced by sapogenins esterified with an acid bearing an extra polar group (i.e. OH or COOH) was compared with that of the corresponding simple esters. It was shown that the presence of a free OH or COOH group in the acid part of the ester inhibits haemolysis. This inhibition may be overcome by acetylation or methylation of the OH or COOH group respectively. It appears that it is not the introduction of an ester group which enhances haemolysis but the removal of the polar group. Sapogenins bearing two polar groups, the esterification of one of them, no matter which, enhances haemolysis, whereas the blocking of the second group reduces this effect. It was further shown that neither the presence of an OH group nor that of any oxygen function at position 3 in the sapogenin is essential for haemolysis.

IN A PREVIOUS report from this laboratory[1] it was stated that the haemolytic effect of the saponins is to be ascribed mainly to their aglyconic component. This finding was supported by the fact that all the sapogenins tested were found to have haemolytic properties comparable to those of the parent saponin.

In contrast to saponins it is relatively easy to obtain pure authentic, even commercial samples of the sapogenins. This, in addition to the above-mentioned findings, enabled us to study the structural requirements for the haemolytic process of this group of compounds. Using these models we were able to show that the haemolytic activity was markedly enhanced by acetylation or benzoylation of the hydroxylic groups as well as by methylation of the carboxylic acid groups. (In the case of glycyrrhetinic acid the increase was as high as 8-fold.)

It may be assumed that a prerequisite of sapogenin haemolysis is the formation of a complex between the sapogenin and a specific receptor on the erythrocyte membrane. Consequently only two possible explanations can be given to the enhancement of haemolysis obtained by esterification of the sapogenin: (a) the esteric function itself increases the extent of haemolysis because of a suitable receptor; (b) the presence of the strong polar groups in the sapogenin interferes with the formation of such a complex with the erythrocyte membrane. In order to investigate these two possibilities we decided to esterify the hydroxyl groups of some sapogenin models with bifunctional acids (i.e. dicarboxylic acids and hydroxy acids). By these means we obtained model compounds in which an ester function as well as a carboxylic acid, or hydroxyl group

were present. The haemolytic activity of these compounds was compared with that of the corresponding simple aliphatic esters (having the same length of carbon chain) or with the corresponding simple aromatic esters. Furthermore a comparison was made with those compounds in which the above-mentioned extra hydroxyl or carboxylic acid groups were removed by acetylation or methylation respectively.

EXPERIMENTAL

Kryptogenin, glycyrrhetinic acid, hecogenin, hecogenin-acetate, tigogenin, tigogenin-acetate and the steroid hormones (5α-androstan-17-one, 5α-androstan-3-β-ol-17-one, 5α-androstan-3β-ol, 5α-androstan-3β-acetoxy-17-one, 5α-androstan-17β-ol) used in this work were commercial products.

The acetates, benzoates, propionates, butyrates, lactates, succinates, phtalates and homophtalates were all synthesized in the same way: the sapogenin was dissolved in a small volume of pyridine and equimolar quantities of the anhydride of the respective acid were added. The mixture was heated for 3 hr at 100–115° and then poured into ice-cold water, filtered and crystallized to constant melting point.

The methyl esters were obtained by methylation of the corresponding acid with diazomethane.

Kryptogenin-26-acetate was obtained by acetylation of kryptogenin with an equimolar quantity of acetic anhydride at room temperature. The desired monoacetate was purified by preparative thick layer chromatography (developing system benzene-7: acetone-3).

Kryptogenin-3-acetate was obtained in three steps, i.e. first tritylation, then acetylation and finally acid hydrolysis to remove the trityl ether. (a) To a 10% solution of kryptogenin in dry pyridine an equimolar quantity of triphenyl chloromethane was added. The mixture was left at room temperature for 3 days then heated for 1 hr at 100°. (b) To the above reaction mixture an equimolar quantity of acetic anhydride was then added and the mixture heated at 120° for 3 hr and then poured into cold water and filtered. (c) The precipitate was then hydrolysed by boiling for 30 min in 1N methanolic HCl, then water was added. The precipitate thus obtained was purified by thick layer chromatography, [developing agent petrol ether (80–100)-7: ethylacetate-3]. Because of the low yield the product was used without further crystallization. The i.r. spectrum of the compound in chloroformic solution shows a band at 3610 cm^{-1} (OH) and at 1736 cm^{-1} (C=O ester).

3-desoxytigogenin was obtained by oxidation of tigogenin to the corresponding ketone and then reduction of the ketone. (a) To 10% solution of tigogenin in acetic acid (distilled over CrO_3), a 2·5% solution of CrO_3 in acetic acid was added until the colour of the chromic acid persisted, then water and sodium bicarbonate were added and the ketone extracted with benzene. The i.r. spectrum of the compound in chloroformic solution shows a band at 1720 cm^{-1} (C=O). (b) Reduction was performed on the crude ketone according to the Huang–Minlon method. The compound was purified on a silica gel column (eluent: benzene-5 $CHCl_3$-5), crystallized from benzene. The i.r. spectrum of the compound shows no-CO band.

Haemolysis

Blood. Citrated rat blood was used. The erythrocytes were separated by centrifugation and washed with an isotonic buffer solution until the supernatant was colourless.

The erythrocytes were then diluted with the same buffer in the proportion of 1:110.

Solutions of haemolysing agents. All the compounds tested were dissolved in dimethyl sulfoxide (DMSO). Care was taken that no precipitation of the solute occurred on dilution with the buffer. The isotonic buffer solution used had the following composition.

$Na_2HPO_4.2H_2O$	3·95g
KH_2PO_4	0·76g
NaCl	7·2g
Aq dist.	1000g

The pH was adjusted to 7·4.

Haemolysis test. To 3·5 ml of the erythrocytes suspension varying amounts of each haemolysing agent were added and the volume was made up to 5 ml with DMSO. The mixture was left at 37° for 2 hr. The optical density of the supernatant obtained after centrifugation was measured at 540 mμ. Complete haemolysis of a standard sample was effected by treatment with water. The H_{50} value (i.e. the concentration giving 50% haemolysis) was determined by plotting percentage haemolysis against concentration. The tolerance of erythrocytes towards dimethyl sulfoxide was tested and found to be 0·3 ml/ml test solution.

RESULTS AND DISCUSSION

Three different types of sapogenins were chosen for the preparation of the model esters: (a) glycyrrhetinic acid—a triterpene sapogenin, (b) hecogenin—a steroidal sapogenin of the spiroketal type, (c) kryptogenin—a steroidal sapogenin with no spiroketal function. These particular sapogenins were chosen because of the marked differences in the haemolysing capacity of the parent compound and that of the corresponding acetates and benzoates.[1] The esterification was carried out with the following bifunctional acids—succinic, phtalic, homophtalic and lactic acids. (Of the lactates only that of glycyrrhetinic acid could be tested since it was extremely difficult to obtain pure compounds of the other lactates, presumably because of the formation of lactoyl lactates.)

The haemolytic potency of the various esters tested is summarized in Table 2.

From the results of Table 2 it becomes obvious that a significant enhancement of haemolysis is only obtained in those cases where the ester function introduced into the sapogenin is a neutral one. Esters with an extra polar group, e.g. the succinates, phtalates, etc., always exhibit a decrease in haemolytic potency as compared with that of the corresponding simple ester (compound 9 with compound 5; 11 with 7; 22 with 19; 24 with 21; 31 with 28; 33 with 30; 35 with 30). It would have been interesting to compare the haemolysis caused by the parent sapogenin with that of the corresponding bifunctional ester, as both possess the same polar groups. This, however, was not possible, in most cases, due to the low solubility of the bifunctional esters. Nevertheless the inhibition of haemolysis caused by the polar groups in the ester was overcome by methylation of the newly-introduced COOH-group or by acetylation of the -OH group, as seen by the comparison of the H_{50} values of the following groups of compounds: 9, 10, 5; 22, 23, 19; 24, 25, 21; 31, 32, 28; 33, 34, 30; 35, 36, 30. (The

TABLE 1. MELTING POINTS AND ANALYSES OF THE ESTERS SYNTHESIZED

Compound	Melting point °C	Analysis			
		Found		Calculated	
		% C	% H	% C	% H
glycyrrhetinic acid lactate	280–281	72·9	8·98	73·07	9·22
glycyrrhetinic acid acetyl lactate	284–285	71·70	8·6	71·92	8·9
glycyrrhetinic acid propionate	273–275	75·09	9·27	75·29	9·5
glycyrrhetinic acid butyrate	244–245	75·58	10·0	75·57	9·63
glycyrrhetinic acid benzoate methyl-ester	307–310	77·1	8·7	77·5	8·84
glycyrrhetinic acid phtalate	238–240	73·91	7·84	73·7	8·1
glycyrrhetinic acid methyl-ester phtalate	314–316	74·1	8·3	73·9	8·1
glycyrrhetinic acid methyl-ester methyl phtalate	229–231	73·99	8·25	74·3	8·36
glycyrrhetinic acid homophtalate	326–328	74·4	8·1	74·0	8·23
glycyrrhetinic acid methyl-ester homophtalate	240–242	73·9	8·4	74·2	8·37
glycyrrhetinic-acid methyl-ester methyl homophtalate	200–204	74·6	8·3	74·4	8·46
kryptogenin di-succinate	165–166	66·1	7·73	66·6	7·94
kryptogenin di-propionate	125–127	73·3	9·46	73·07	9·23
kryptogenin di-butyrate	99–103	73·78	9·45	73·69	9·48
kryptogenin di-phtalate	206–208	72·1	7·8	72·6	7·9
kryptogenin 26-acetate	130–133	74·0	9·30	73·7	9·33
hecogenin succinate	221–223	70·6	8·4	70·1	8·6
hecogenin succinate methyl-ester	172–174	70·3	8·6	70·5	8·8
hecogenin propionate	231–235	74·0	9·7	74·07	8·69
hecogenin butyrate	194–197	74·1	9·7	74·4	9·8
hecogenin benzoate	265–268	76·1	8·4	76·3	8·6
hecogenin phtalate	230–231	72·9	7·8	72·6	7·9
hecogenin phtalate methyl-ester	193–195	72·7	7·9	72·9	8·1
hecogenin homophtalate	233–236	73·0	8·1	72·9	8·1
hecogenin homophtalate methyl-ester	200–202	73·3	8·0	73·2	8·2
3-desoxy-tigogenin	185–190				

groups of compounds 12, 13, 8 and 15, 16, 8 are exceptions to which we shall refer later.)

From the above we may conclude unequivocally that the enhancement of haemolysis by acetylation of a hydroxyl group or by methylation of a carboxylic acid group in the sapogenin is *not* effected by the introduction of an ester function but by the absence of the said polar functions.

Further, it can be seen that there is no significant difference between H_{50} values of the acetates, propionates and butyrates. Esters with higher fatty acids could not be tested because of their low solubility. Therefore no general conclusions can be drawn about the effect which the length of the aliphatic chain in the acid part of the ester has on haemolysis.

The problem which next arose was whether we may ascribe the "ester effect" to the low polarity of the sapogenin esters as compared to that of the parent compound. If this is correct then the total removal of the -OH group from the sapogenin molecule should greatly increase haemolysis. In order to investigate this we compared the H_{50} values of two sets of compounds: tigogenin, tigogenin-acetate, 3-desoxy-tigogenin (43, 44, 45) and 5α-androstan-3β-ol-17-one, 5α-androstan-3β-acetoxy-17-one, 5α-androstan-17-one (46, 47, 48). From these results (Table 2) it is clear that in steroids

TABLE 2. HAEMOLYTIC POTENCY (EXPRESSED AS H_{50}) OF SAPOGENINS AND THEIR ESTER DERIVATIVES

No. of compound	Name of compound	H_{50} γ/cm²
1	glycyrrhetinic acid	93
2	glycyrrhetinic acid methyl ester	10·5
3	glycyrrhetinic acid acetate	5·3
4	glycyrrhetinic acid acetate methyl ester	24
5	glycyrrhetinic acid propionate	8
6	glycyrrhetinic acid butyrate	4·6
7	glycyrrhetinic acid benzoate	8·5
8	glycyrrhetinic acid benzoate methyl ester	35
9	glycyrrhetinic acid lactate	21·6
10	glycyrrhetinic acid acetyl lactate	7·2
11	glycyrrhetinic acid phthalate	> 100*
12	glycyrrhetinic acid methyl ester phthalate	33·2
13	glycyrrhetinic acid methyl ester methyl phthalate	39·3
14	glycyrrhetinic acid homophthalate	77·2
15	glycyrrhetinic acid methyl ester homophthalate	18·2
16	glycyrrhetinic acid methyl ester methyl homophthalate	61
17	kryptogenin	111
18	kryptogenin-3, 26-diacetate	15
19	kryptogenin-3, 26-dipropionate	26
20	kryptogenin-3, 26-dibutyrate	7·7
21	kryptogenin-3, 26-dibenzoate	9·9
22	kryptogenin-3, 26-disuccinate	> 500*
23	kryptogenin-3, 26-disuccinate dimethyl ester	71·6
24	kryptogenin-3, 26-diphthalate	> 100*
25	kryptogenin-3, 26-diphthalate dimethyl ester	44
26	hecogenin	> 25*
27	hecogenin acetate	3·4
28	hecogenin propionate	5·4
29	hecogenin butyrate	5·2
30	hecogenin benzoate	9·5
31	hecogenin succinate	> 25*
32	hecogenin succinate methyl ester	1·6
33	hecogenin phthalate	> 25*
34	hecogenin phthalate methyl ester	6·8
35	hecogenin homophthalate	> 25*
36	hecogenin homophthalate methyl ester	5·2
37	kryptogenin-3 acetate	30
38	kryptogenin-26 acetate	19
39	oleanolic acid	> 100*
40	oleanolic acid acetate	6·2
41	oleanolic acid methyl ester	2·5
42	oleanolic acid acetate methyl ester	8
43	tigogenin	10·4
44	tigogenin acetate	7·1
45	3-desoxy-tigogenin	12
46	5α-androstane-3β-ol-17-one	114
47	5α-androstane-3βacetoxy-17-one	17·7
48	5α-androstane-17-one	0·8
49	5α-androstane-3β-ol	3·9
50	5α-androstane-17β-ol	5·5

* Maximum concentration obtainable.

neither an -OH group nor an ester group at position 3 is essential for haemolysis. (In our opinion the haemolysis caused by the androstan model is comparable with that of the steroidal sapogenins, since it is the steroid part of the saponin molecule which is responsible for haemolysis and since the androstan derivative has the 5α configuration similar to that of the sapogenins tested.)

The two sets of samples tested do not yield sufficient evidence to determine whether

the 3-acetoxy- or the 3-desoxy-steroids are more potent haemolysins. However, they show clearly that neither the -OH nor any other oxygen function at position 3 are essential for haemolysis.

In the course of our investigations our attention was drawn to the interesting behaviour of those sapogenins, such as glycyrrhetinic acid, oleanolic acid and kryptogenin, which have two polar groups. In these sapogenins it was seen that the introduction of the first esteric function markedly enhances haemolysis whereas esterification of *both* polar groups has only little, or even an adverse effect. Comparison of the following groups of compounds points this out (Table 2): 1, 2, 3, 4; 1, 2, 7, 8; 39, 40, 41, 42; 20, 37, 38, 18. Further, if glycyrrhetinic acid phtalate and homophtalate are regarded as simple bifunctional sapogenins, then the following two sets of compounds, 14, 15, 16 and 17, 18, 19, can be added. In this light then the behaviour of compounds 15 and 19 (as discussed previously) no longer appeared to be exceptional.

Similar results were obtained by Schlösser and Wulff in their investigation of the structural specificity of triterpene induced haemolysis.[2] Since these authors make no distinction between the behaviour of mono and diesters, their interpretation of the results differs completely from ours. Comparing the activity of the methyl esters of 3-hydroxy triterpene acids with that of the corresponding 3-acetoxy methyl esters, brought them to the conclusion that a 3-hydroxy function is essential for haemolysis, and that acetylation at this position greatly inhibits it. This assumption is claimed to be supported by the results obtained by investigating four other simple sapogenin acetates. Three of these, however, are esters of sapogenins which themselves are non-haemolytic (compounds 2, 5, 9—Table on page 1286 of the above reference), so that no comparison is possible. The only example which apparently supports this statement is that of echinocystic acid and its acetate (cf. 17 and 17a). This exceptional result may probably be ascribed to the fact that they did not test whether the sapogenin precipitated out of the DMSO solution when it was added to the aqueous erythrocyte suspension. It seems doubtful, therefore, to us whether their results point unequivocally to the conclusions drawn by them.

Another fact that should be pointed out is that in those bifunctional sapogenins tested it is of minor importance only which polar group is esterified, *i.e.* the position and the nature of the group is of little significance. For example, among the derivatives of glycyrrhetinic acid the activity of the acetate is only twice that of the methyl derivative, but the acetate is 20 times more haemolytic than the parent sapogenin. The same general behaviour was observed with the other bifunctional sapogenins tested, i.e. oleanolic acid and kryptogenin.

As yet we cannot explain why, in our models, the place of attachment of the ester group has so little effect on haemolysis and also why the diesters of the bifunctional sapogenins are less haemolytic than the monoesters. Possibly, when more is known about the structural requirements for haemolysis, these facts will be explained. However, the only fact which is well established is that the esterification of a sapogenin (either steroid or triterpene) with a non-polar acid or alcohol enhances the haemolytic properties because of the elimination of the polar groups from the parent molecule. The question if sapogenin induced haemolysis may be correlated with the haemolytic property of steroidal hormones,[3–6] is now under investigation.

Acknowledgements—This work was supported by a grant from the Haematology Research Foundation, Chicago.

We thank "Ikapharm-Pharmaceutical Laboratories" for the generous gift of the steroidal hormones.

REFERENCES

1. R. Segal, M. Mansour and D. V. Zaitschek, *Biochem. Pharmac.* **15**, 1411 (1966).
2. E. Schlösser and G. Wulff, *Z. Naturforsch.* **24b**, 1284 (1969).
3. J. Tateno and E. D. Kilbourne, *Proc. Soc. exp. Biol. Med.* **86**, 168 (1954).
4. R. H. Palmer, *Nature, Lond.* **201**, 1134 (1964).
5. A. Karpas and R. H. Palmer, *Pharmac. Rev.* **15**, 123 (1963).
6. G. Weissmann and H. Keiser, *Biochem. Pharmac.* **14**, 537 (1965).