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Structure–Activity Relationships in the Field of Antibacterial Steroid Acids

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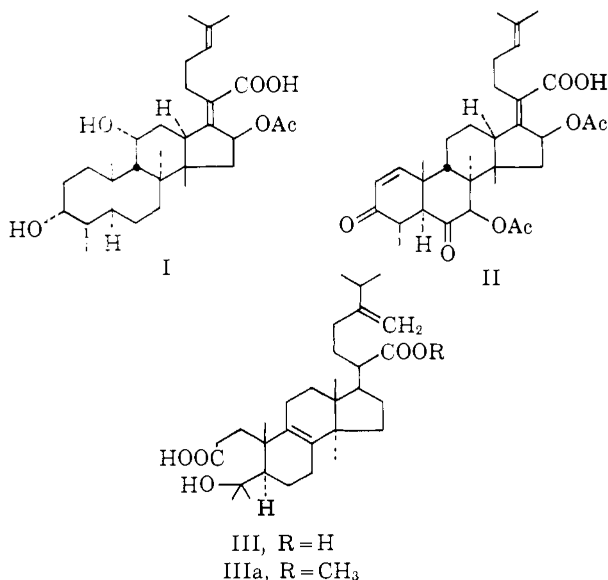
The antibacterial activity of a variety of steroidal and triterpenoid acids has been determined using *Staphylococcus aureus* 209P as the test organism. Activity was found to be less dependent on specific structural and stereochemical features than had been anticipated. All active compounds have a rigid polycyclic skeleton with a carboxyl group close to an oxygen function or a double bond.

Recent investigations leading to the establishment of the structure of the antibiotics fusidic acid (I),[2] helvolic acid (II),[3,4] and cephalosporin P_1[5] as tetracyclic triterpenoids closely related to the steroids have documented the biological versatility of this important nucleus in yet another area. The subsequent conversion of the structurally related triterpenoid acid eburicoic acid into an antibacterially active ring A seco-acid (III) by means of microbial enzymes[6] has prompted us to undertake a broader investigation of the antibacterial properties of steroidal and triterpenoid acids. It seemed at the outset that, because of the considerable differences in the stereochemistry between the naturally occurring antibiotics and the seco-acid III, the structural requirements for antibacterial activity might not prove to be too exacting. This impression gained further support when considering the fact that, while in the steroid antibiotics the important acidic function is located at C-21 and forms part of the side chain, the biological activity of the seco-acid III is dependent on the presence of a carboxyl group at C-3 as demonstrated by the increase in activity attending methylation of the 21-carboxyl group.[7] The above impressions have been fully borne out by the data reported in this paper.



It appeared most appropriate to begin such a study by preparing additional ring A seco-acids from a variety of sterols and triterpenes, and to assess the influence of the rigid portion of the steroid nucleus and of the side chain on biological activity. These data are summarized in Table I. With the exception of compounds **1**, **2**, and **3**, all those listed in this table possess the *anti-trans-anti* backbone characteristic of the sterols. However, the location of the angular methyl groups varies widely and so does the nature of the side chain. With the exception of compound **8**, all of the compounds were active and one can conclude from these data that considerable structural variation is possible without loss of antibacterial activity. This is particularly evident when one considers the lupeol derivative **7**, which shows the most far-reaching deviation in skeletal structure. No attempt is being made here to evaluate the quantitative differences in activity between the various compounds except in those cases where only a single substituent is being varied. This is the case for **2**, the 21-methyl ester of **1**, which shows a 30-fold enhancement in activity over the latter. The conclusion appears justified that a second anionic site at a distant part of the molecule is detrimental to activity. It is felt, on struc-

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(4) S. Okuda, S. Iwasaki, K. Tsuda, Y. Sano, T. Hata, S. Udagawa, Y. Nakayama, and H. Yamaguchi, *Chem. Pharm. Bull. Japan*, **12**, 121 (1964). Structure II was proposed by Professor S. Okuda at the Symposium on the Chemistry of Natural Products, Nagoya, Oct. 1964; *cf.* Abstracts of Papers, p. 192.

(5) B. M. Baird, T. G. Halsall, E. R. H. Jones, and G. Lowe, *Proc. Chem. Soc.*, 257 (1961); 16 (1963).

(6) A. I. Laskin, P. Grabowich, C. de Lisle Meyers, and J. Fried, *J. Med. Chem.*, **7**, 406 (1964).

(7) The necessity for the presence of at least one carboxyl group derives from the fact that the dimethyl ester of III was inactive at the 100-γ/ml. level, the highest level employed in this investigation. Similarly, reduction of both carboxyl groups to primary alcohols led to inactive compounds; *cf.* ref. 6.

TABLE I

VARIATIONS IN SKELETAL STRUCTURE AND SIDE CHAIN

Compd.	Structure	MIC,[a] γ/ml.
1[b]		60
2[c]	21-Methyl ester of 1	2
3[b]	24(28)-Dihydro-21-methyl ester of 1	2
4[c]		9
5[c]		9
6[d]		75
7[e]		50
8[d]		> 50
9[e]	Fusidic acid	0.02

[a] Minimum inhibitory concentration of steroid acids in two-fold serial dilution assay against *S. aureus* 209P. [b] Ref. 6. [c] This work. [d] Ref. 8. [e] Ref. 2.

TABLE II

RING A SECO-ACIDS POSSESSING THE CHOLESTANE SKELETON

Compd.	Structure	MIC,[a] γ/ml.
1[b]		75
2[c]		30
3[b,c]		7
4[b,c]		60
5[d]		80
6[e]		>100
7[f]		70
8[g]		>100
9[h]		2
10[b]		>100
11[e]		>100
12[b]		>100

[a] Minimum inhibitory concentration of steroid acids in two-fold serial dilution assay against *S. aureus* 209P. [b] Ref. 8. [c] V. Burckhardt and T. Reichstein, *Helv. Chim. Acta*, **25**, 821, 1434 (1942). [d] L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p. 41. [e] Ref. *d*, pp. 77, 80. [f] Ref. *d*, pp. 63, 80. [g] Ref. *d*, p. 80. [h] Ref. *d*, p. 74.

tural grounds, that **8** which lacked activity at 50 γ might have been active when tested at a higher level. But this is to be expected if an arbitrary upper limit is set for testing.

Table II summarizes our data on ring A seco-acids, in which substituents attached to "ring A" are varied and the remaining portion of the steroid skeleton is that of cholestane. Since the hydroxy acids **1–4** all showed activity, the degree of methylation at C-4 has little influence on activity. "Compounds" **3** and **4** represent approximately equimolar mixtures of the two isomeric hydroxy acids shown in Table II.[8] At least one of the two, if not both isomers, must therefore, be considered active. Among the dicarboxylic acids only the two *trans*-acids **5** and **7** showed activity, the two *cis*-acids being inactive at the 100-γ level. An interesting contrast in activity was shown between the well-known keto acid **9** and its 5-deoxo derivative **10**. The former was active at the 2-γ level, whereas the latter was inactive at 100 γ. The presence of an oxygen function in the γ- or δ-position with respect to the carboxyl group has been a characteristic feature of all active compounds discussed so far, and, indeed, it is a characteristic of the naturally occurring steroid antibiotics, as well. The

(8) D. Rosenthal, A. O. Niedermeyer, and J. Fried, *J. Org. Chem.*, **30**, 510 (1965).

lack of activity of compound **10** indicates that this may be an essential requirement for antibacterial activity. As we shall see later, a double bond appropriately located may take the place of this oxygen function. The simple alicyclic δ-keto acid, cyclohexanone-2-(3-propionic acid) (Table III, **2**), was inactive, stressing the necessity for a larger condensed ring system. This latter condition is fulfilled in the ring B seco-acid (Table III, **3**), which showed activity equal to that of the keto acid **9** (Table II).

TABLE III

MISCELLANEOUS ACIDS

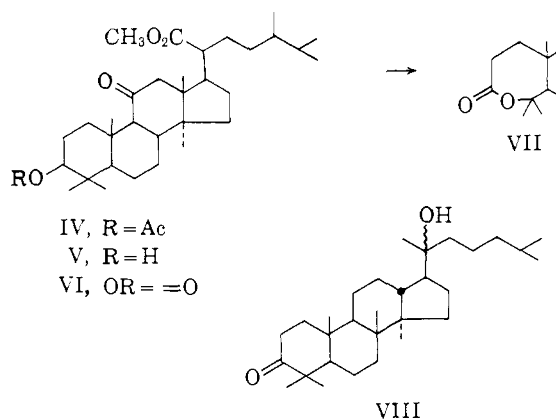
Compd.	Structure	MIC,[a] γ/ml.
1[b]	HO, HO, CH_2OH, CO_2H	14
2[c]	HO_2C, O	>100
3	HO, O, COOH	2–3
4[d]	HO_2C	1–2
5[e]	HO_2C	>100

[a] Minimum inhibitory concentration of steroid acids in twofold serial dilution assay against *S. aureus* 209P. [b] J. Polonsky, *Bull. soc. chim. France*, 173 (1953); S. Brewis and T. G. Halsall, *J. Chem. Soc.*, 646 (1961); *cf.* also ref. 11. [c] V. V. Korshak, S. L. Sosin, and E. M. Morozova, *Zh. Obshch. Khim.*, **30,** 907 (1960); *Chem. Abstr.*, **55,** 376c (1961). [d] L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p. 55; [e] Ref. *d*, p. 61.

Unexpectedly high activity (1–2 γ/ml.) was shown by cholatrienic acid (Table III, **4**). Other bile acid derivatives such as the fully saturated cholanic acid (Table III, **5**), 3,12-diketocholanic acid, and 3,7,12-triketocholanic acid were inactive. Perhaps, the 11,12-double bond of cholatrienic acid takes the place of the oxygen function considered necessary for activity, but this is a rather tenuous assumption, because of the distance of the double bond from the carboxyl group and because of the fact that the two 12-keto acids tested did not show activity. The triterpenoid asiatic acid (Table III, **1**), which possesses a double bond in the γ-position with respect to the (axial) carboxyl group, may more justifiably be viewed as an analog of the active γ- or δ-hydroxy or keto acids, in which the double bond takes the place of the oxygen function.

The data presented here although admittedly sketchy indicate that the antibacterial activity of the steroid acids is a phenomenon of limited structural and steric specificity. What appears to be necessary is a rigid, essentially flat molecule possessing at least, and preferably, one anionic site, which is close to an oxygen function or perhaps also the π-electrons of a double bond. These requirements are fulfilled in the naturally occurring steroid antibiotics, all of which possess an acetoxy group in γ-position to the carboxyl group. It should be kept in mind that the most active compounds described in this paper possess about 1/50 to 1/100th the activity of fusidic acid, indicating that our conclusions regarding the limited structural requirements for activity will have to be refined when it comes to define *maximum* activity. These more subtle factors, however, may no longer be related to intrinsic biological activity, that is, the chemical events occurring at the site of action, but may be connected with factors such as transport to the site of action, metabolic inactivation, complexing with cell constituents, etc. The attainment of maximum biological activity represents a sequence of events of such complexity that, to consider our data in a more quantitative sense would be all but meaningless. Nevertheless, by defining the minimum requirements for activity, the findings presented in this paper should prove useful in the selection of additional antimicrobially active compounds from among known steroids and terpenes, and in the synthesis of new, hopefully more active, representatives of this class.

The validity of the above generalizations regarding the relationships of structure and activity among antimicrobial steroids is dependent upon a common mechanism of action for both the naturally occurring antibiotics and the steroidal acids reported in this paper. Such correspondence was demonstrated by showing that a strain of *S. aureus* made resistant towards fusidic acid was also resistant to the action of two synthetic compounds representative of this work (Table I, **2**, and Table II, **3**). Both were inactive at 100 γ/ml. when tested against that strain.



Experimental

Melting points were taken on a Thomas-Hoover apparatus and are corrected for stem exposure. Rotations were determined in chloroform. Infrared spectra were taken on a Perkin-Elmer 21 spectrometer.

The substances listed in Tables I–III were either taken from our collection of steroids or were prepared specifically for the purpose of this paper. Those not hitherto described in detail

are described in the following. Structures IV–VIII represent intermediates used in the preparation of the final products.

Methyl Eburican-3β-ol-11-on-21-oate (V).—A solution of 2.0 g. of methyl eburican-3β-ol-11-on-21-oate 3-acetate (IV)[9] in 400 ml. of 1 N ethanolic KOH was allowed to stand at room temperature for 18 hr. The solution was neutralized with 20% H_2SO_4 and diluted with water, and the ethanol was evaporated. The aqueous suspension was extracted with ether, and the ether solution was washed with water, dried ($MgSO_4$), and evaporated to dryness. Recrystallization from methanol gave 1.55 g. of V, m.p. 133–135°. The analytical sample melted at 137–139°; $[\alpha]^{25}D$ +32°; λ_{max}^{Nujol} 2.97, 5.77, 5.89 μ.

Anal. Calcd. for $C_{32}H_{54}O_4$: C, 76.44; H, 10.83. Found: 76.14; H, 10.63.

Methyl Eburicane-3,11-dion-21-oate (VI).—A solution of 778 mg. of methyl eburican-3β-ol-11-on-21-oate (V) in 55 ml. of acetone was treated with a 10% excess of Jones reagent at room temperature. The excess chromic acid was decomposed with methanol, the reaction mixture was diluted with 25 ml. of water, and the organic solvent was evaporated *in vacuo*. The aqueous suspension was extracted with chloroform, and the chloroform solution was washed with water, dried, and evaporated to give 784 mg. of crude product. This was adsorbed on a column of 25 g. of neutral alumina and eluted with benzene and benzene–chloroform (9:1) to give 734 mg. of VI, which on recrystallization from methanol gave a first crop of 443 mg., m.p. 139–140°, and a second crop of 152 mg., m.p. 137–139°. The analytical sample had m.p. 143–144°, $[\alpha]^{25}D$ +42°, λ_{max}^{Nujol} 5.77 and 5.87 μ.

Anal. Calcd. for $C_{32}H_{52}O_4$: C, 76.75; H, 10.47. Found: C, 76.77; H, 10.43.

3,4-Secoeburican-4-ol-11-one-3,21-dioic Acid 21-Methyl Ester (3→4)-Lactone (VII).—A solution of 242 mg. of methyl eburicane-3,11-dion-21-oate (VI, 0.5 mmole) and 345 mg. of *m*-chloroperbenzoic acid (2 mmoles) in 5 ml. of methylene dichloride was kept at room temperature for 43 hr. The reaction mixture was diluted with methylene dichloride and washed successively with 5% KI, 5% sodium sulfite, water, 5% $KHCO_3$, and water, dried, and evaporated to dryness to give 246 mg. of crude product. Two recrystallizations from methanol afforded 134 mg. of the lactone VII, m.p. 167–170°, $[\alpha]^{23}D$ +70°, λ_{max}^{KBr} 5.78 and 5.85 μ.

Anal. Calcd. for $C_{32}H_{52}O_5$: C, 74.37; H, 10.14. Found: C, 74.50; H, 10.32.

3,4-Secoeburican-4-ol-11-one-3,21-dioic Acid 21-Methyl Ester (Table I, 4).—A solution of 100 mg. of the above lactone VII in 10 ml. of 5% methanolic KOH was kept at room temperature for 6 hr. The pH was adjusted to 4.5 and the reaction mixture was diluted with water. The methanol was evaporated, the aqueous suspension was extracted with chloroform, and the chloroform solution was washed with water, dried, and evaporated. The crude acid was chromatographed on silica gel, and the chloroform and chloroform–acetone (1:1) eluates were combined and evaporated to give 39 mg. of product (Table I, 4).

Anal. Calcd.: neut. equiv., 535. Found: neut. equiv., 505.

3,4-Secolupan-4-ol-3-oic Acid (3→4)-Lactone.—A solution of 0.426 g. of lupan-3-one (1 mmole, m.p. 206–207°)[10] and 1.382 g. of *m*-chloroperbenzoic acid (8 mmoles) in 10 ml. of chloroform was kept at room temperature for 23 hr. Upon work-up as described above, material was obtained, which after two recrystallizations from acetone gave 166 mg. of the lactone of 3,4-secolupan-4-ol-3-oic acid (VII), m.p. 193–196°, $[\alpha]^{20}D$ + 35°, λ_{max}^{Nujol} 5.79 μ.

Anal. Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38. Found: C, 81.40; H, 11.38.

3,4-Secolupan-4-ol-3-oic Acid (Table I, 7).—A suspension of 102 mg. of the above lactone was hydrolyzed with 30 ml. of 5% ethanolic KOH for 6 hr. The crude product (105 mg.) after two crystallizations from acetone afforded 43 mg. of analytically pure 3,4-secolupan-4-ol-3-oic acid (Table I, 7), m.p. 210–212°, λ_{max}^{Nujol} 3.30 and 5.82 μ, $[\alpha]^{25}D$ −0.8°.

Anal. Calcd. for $C_{30}H_{52}O_3$: C, 78.20; H, 11.38; neut. equiv., 460. Found: C, 78.08; H, 11.48; neut. equiv., 457.

3,4-Secodammarane-4,20ξ-diol-3-oic Acid (Table I, 5).—A solution of 200 mg. of 24,25-dihydrodipterocarpol (hydroxydammaranone-II, VIII, 0.43 mmole, m.p. 97–98°)[11] and 600 mg. of *m*-chloroperbenzoic acid (3.45 mmoles) in 5 ml. of chloroform was kept at 25° for 23 hr. The resulting crude product on thin layer chromatography on neutral alumina (activity V) in a hexane–chloroform (1:4) system and elution of the area R_f 0.40–0.60 gave 131 mg. of the lactone of 5 (Table I). The total crude lactone was hydrolyzed with 20 ml. of 5% ethanolic KOH at room temperature for 6.5 hr., and the isolated acid was recrystallized from methanol. There was obtained 85 mg. of compound 5 (Table I), m.p. 175.5–176°, λ_{max}^{KBr} 2.93 and 5.85 μ, $[\alpha]^{25}D$ +40°.

Anal. Calcd. for $C_{30}H_{54}O_4$: C, 75.26; H, 11.37; neut. equiv., 478. Found: C, 75.19; H, 11.26; neut. equiv., 457.

Bioassay Procedure.—The *in vitro* antimicrobial activity was determined by a twofold serial tube dilution assay in Penassay broth. The test organism, *S. aureus* 209 P, was grown overnight in Penassay broth and a 10^{-6} dilution of this culture was used as the seed inoculum. The steroid was added as a 1% solution in 50% dimethyl sulfoxide. After 18–24-hr. incubation at 37°, the minimum inhibitory concentration (MIC) was determined from the tube containing the least amount of steroid, in which no visible growth could be observed.

(9) G. W. Krakower, J. W. Brown, and J. Fried, *J. Org. Chem.*, **27**, 4710 (1962).

(10) I. M. Heilbron, T. Kennedy, and F. S. Spring, *J. Chem. Soc.*, 329 (1938). This sample was prepared by catalytic hydrogenation of lupenone, a gift from Dr. O. Wintersteiner who isolated this substance from *Samadera indica*.

(11) T. S. Mills, *J. Chem. Soc.*, 2196 (1956). This substance was prepared from dipterocarpol, isolated from the resin of *Dipterocarpus borneensis* obtained through the courtesy of The Tropical Research Council, London.