PREPARATION AND ANTIBACTERIAL ACTIVITY OF DI-, TRI- AND TETRAOIC ACIDS DERIVED FROM 3,4-SECOLUPANE*

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Diacids *XXIII* and *XXIV*, triacid *XXVII* and tetraacid *XVI* were prepared from 3,4-secodiacid *V* and from nitrile *XVII* by modifying the isopropenyl groups in positions 5 and 19. Procedures for selective esterification of the acids and the hydrolysis of their esters in positions 3, 23 and 28 were elaborated and the methyl esters *VII*, *XI*, *XII*, *XXVI*, *XXVIII*, *XXX* and *XXXI* prepared. The structures of the derivatives prepared were confirmed by mass spectra. The antibacterial activity of the series of 3,4-secoacids derived from lupane and 19β,28-epoxy-18α-oleanane was also determined.

In our preceding paper[1] we described the preparation of 3,4-secolupane-3,28-dioic acids with an oxygen-containing functional group in the side chain on $C_{(5)}$, which fulfil the structural requirements for antibacterial activity (oxygen-containing function or a double bond in the vicinity of the carboxyl group, see[2]). This study is devoted to the preparation of di-, tri- and tetraoic acids derived from 3,4-secolupane skeleton, which have a double bond in the side chain at $C_{(5)}$ (or also in the chain at $C_{(19)}$) in addition to the oxygen-containing group.

Starting materials were diacid *V* and nitrile *XVII*, obtained from betulinic acid (*I*) in a manner described[3]. The preparation of acid *V* was carried out without the purification of intermediates *II*, *III* and *IV* and it was modified for larger scale work. Thus diacid *V* was obtained from acid *I* in a 52% total yield. For the introduction of the functional groups into the side chains on $C_{(5)}$ and $C_{(19)}$ the methods described in literature[4,5] were used.

For the preparation of some derivatives which fulfil the conditions necessary for antibacterial activity, formulated by Fried and coworkers[2], methods for selective esterification of carboxyl groups in various parts of the 3,4-secolupane molecule had to be developed. Therefore preliminary model experiments were carried out with the unsaturated acid *V* and the tetrahydrodiacid *IX* (prepared according to ref.[3]). The carboxyl groups in position 3 and 28 differ in their reactivity, which was

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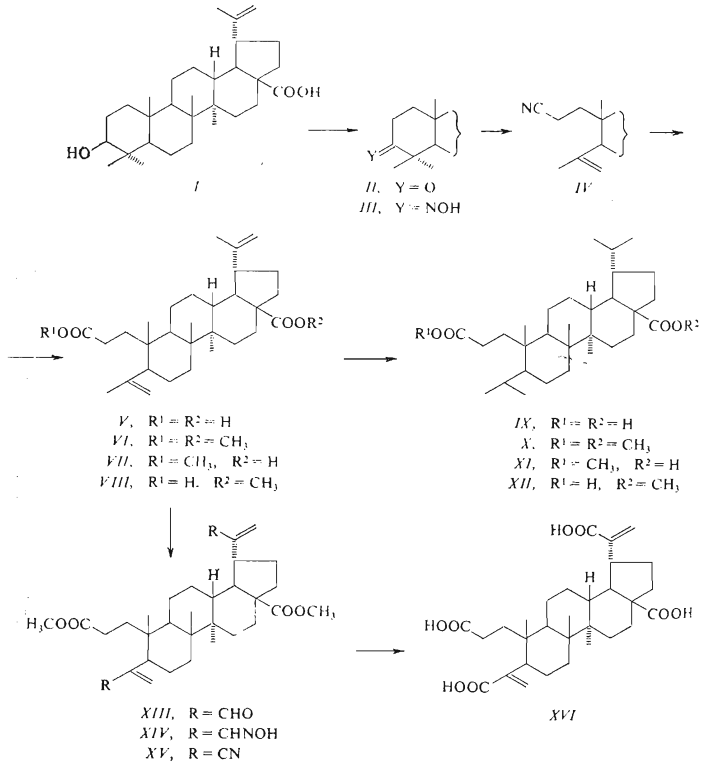
made use of earlier[3] for the preparation of monomethyl ester *VIII* by partial hydrolysis of dimethyl ester *VI*. 3-Monomethyl ester *VII* has now been prepared by boiling diacid *V* in methanol in the presence of Dowex cation exchanger. Under these conditions no undesirable rearrangements[6,7] take place and a selective esterification of the carboxyl group in position 3 proceeds smoothly. Analogously, the reaction of tetrahydrodiacid *IX* with methanol in the presence of the Dowex catalyst afforded 3-monomethyl ester *XI*. 28-Monomethyl ester *XII* was prepared by partial hydrolysis of dimethyl ester *X* with 2% potassium hydroxide in a mixture of benzene and methanol.



I

II, Y = O
III, Y = NOH

IV

V, $R^1 = R^2 = H$
VI, $R^1 = R^2 = CH_3$
VII, $R^1 = CH_3$, $R^2 = H$
VIII, $R^1 = H$, $R^2 = CH_3$

IX, $R^1 = R^2 = H$
X, $R^1 = R^2 = CH_3$
XI, $R^1 = CH_3$, $R^2 = H$
XII, $R^1 = H$, $R^2 = CH_3$

XIII, R = CHO
XIV, R = CHNOH
XV, R = CN

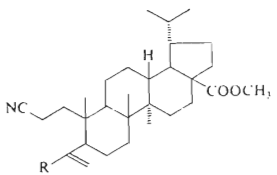
XVI

The reaction of unsaturated dimethyl ester *VI* with selenium dioxide in acetic acid, under the conditions given in literature[5], gave α,β-unsaturated dialdehyde *XIII*. Its reaction with hydroxylamine hydrochloride led to dioxime *XIV* which was dehydrated in acetic anhydride under formation of dinitrile *XV*. Hydrolysis of dinitrile *XV* with 10% sodium hydroxide in boiling ethylene glycol gave unsaturated tetraacid *XVI*.

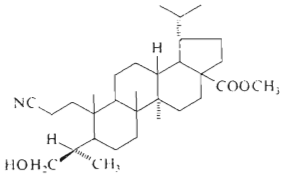
Further derivatives were obtained from nitrile *XVII*. On oxidation with selenium dioxide, carried out in the same manner as in the preceding case, it gave α,β-unsaturated aldehyde *XVIII*. From aldehyde *XVIII* two series of seco derivatives were prepared. The first one contains the hydroxyl group or the acetoxy group in α-position to the double bond, while the second is characterized by an α,β-unsaturated carboxyl group or a methoxycarbonyl group in the side chain on $C_{(5)}$. On reduction of aldehyde *XVIII* with sodium borohydride hydroxy nitrile *XIX* was obtained. As a by-product the saturated hydroxy nitrile *XXII* was formed, with a 4*R* configuration (see[1]), which was also observed in a similar reduction in the 18α-oleanane series[4]. Under the effect of a 10% sodium hydroxide solution in ethylene glycol hydroxy nitrile *XIX* gave hydroxy diacid *XXIII* which reacted with acetic anhydride in pyridine to acetate *XXIV* (the reaction product consisted of a mixture of free acid *XXIV* and mixed anhydride with acetic acid; the anhydride could be hydrolysed by boiling in aqueous dioxane, where the acetoxy group remained preserved). Reaction of hydroxy diacid *XXIII* with diazomethane gave dimethyl ester *XXV* which was partially hydrolysed with 0·2% potassium hydroxide to 28-monoester *XXVI*.

The second series of reactions starting with aldehyde *XVIII* led to triacid *XXVII* and its esters. Analogously as in the preparation of tetraacid *XVI* the oxime *XX* was prepared from aldehyde *XVIII* and then dehydrated to nitrile *XXI*, which was hydrolysed with 10% sodium hydroxide in ethylene glycol to triacid *XXVII*. According to the analogy with model experiments 3-monomethyl ester *XXVIII* was prepared from triacid *XXVII* by esterification with methanol in the presence of Dowex ion exchanger. When reacting with diazomethane triacid *XXVII* afforded trimethyl ester *XXIX* which was partially hydrolysed in two ways, affording thus esters *XXX* and *XXXI*. 23,28-Dimethyl ester *XXX* was prepared on hydrolysis under mild conditions (boiling with 2·5% potassium hydroxide in a mixture of benzene and methanol), while stronger conditions of hydrolysis (6 hours' boiling with a 2% solution of sodium hydroxide in aqueous dioxane) led to the formation of 28-monomethyl ester *XXXI*. From the course of the partial esterification and hydrolysis it follows that the carboxyl group or the ester group in position 3 is much more reactive than the same group conjugated with the double bond.

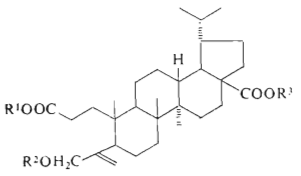
The structure of the di-, tri-and tetraacids and their esters prepared and the position of the ester groups in partially esterified derivatives of diacid *IX* and triacid *XXVII* was further confirmed by mass spectra. The interpretation is based on the fragmenta-



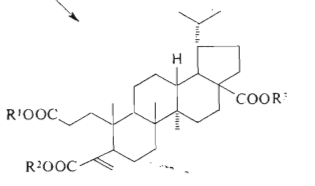
XVII, R = CH_3
XVIII, R = CHO
XIX, R = CH_2OH
XX, R = CHNOH
XXI, R = CN



XXII



XXIII, $R^1 = R^2 = R^3 = H$
XXIV, $R^1 = R^3 = H$, $R^2 = COCH_3$
XXV, $R^1 = R^3 = CH_3$, $R^2 = H$
XXVI, $R^1 = R^2 = H$, $R^3 = CH_3$



XXVII, $R^1 = R^2 = R^3 = H$
XXVIII, $R^1 = CH_3$, $R^2 = R^3 = H$
XXIX, $R^1 = R^2 = R^3 = CH_3$
XXX, $R^1 = H$, $R^2 = R^3 = CH_3$
XXXI, $R^1 = R^2 = H$, $R^3 = CH_3$

tion mechanisms described for 3,4-secotriterpenes by Aplin and Cox[8]. The characteristic ions are listed in Table I. As the most important fragments the ions $[M-a]^+$ were found which are formed by the splitting off of the substituent in the position 17β and a hydrogen atom ($a = HC_{(28)}OOR$, where R is H or CH_3), and the ions $[M-b]^+$, formed by loss of the side chain from $C_{(10)}$, comprising the atoms $C_{(1)}$ to $C_{(3)}$ ($b = {}^{\bullet}C_{(1)}H_2C_{(2)}H_2C_{(3)}OOR$, where R is H or CH_3). The third basic fragment is the ion $[M-c]^+$, which, according to ref.[8], is formed by the cleavage of the bonds $C_{(5)}$—$C_{(10)}$ and $C_{(7)}$—$C_{(8)}$ under hydrogen transfer from position 6 to position 10 and elimination of the fragment containing the atoms $C_{(5)}$ to $C_{(7)}$ and the side chain ($c = R-C_{(5)}H=C_{(6)}HC_{(7)}H_2^{\bullet}$, where R is the side chain on $C_{(5)}$). The ions which are formed by combined fragmentation also appear in the spectrum.

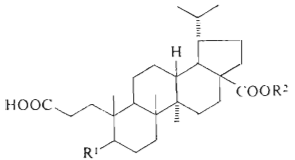
as for example $[M-a-b]^+$ and $[M-a-c]^+$. The ions $[M-a]^{+\cdot}$, $[M-b]^+$ and $[M-a-b]^+$ are present in the spectra of all the substances measured. In contrast to this the ions $[M-c]^+$ and $[M-a-c]^+$ are characteristic only of derivatives with a double bond in the side chain on $C_{(3)}$ (compounds *XVI*, *XXVII*–*XXXI*), while in the spectra of saturated derivatives *IX*–*XII* they do not appear. In free 28-acids *IX*, *XI*, *XXVII*, *XXVIII* another characteristic ion, *m/z* 261, was found which is probably formed on splitting of the skeleton between the rings B and C, and in analogy with ref.[9] it might by rationalized as *d*. In 28-methyl esters *X*, *XII*, *XXIX*–*XXXI* the ion *m/z* 275 corresponds to it, and in tetraacid *XVI* the ion *m/z* 289.

Eight of the prepared 3,4-secoacids fulfil the conditions for antibacterial activity[2] (*XVI*, *XXIII*, *XXIV*, *XXVI*–*XXVIII*, *XXX* and *XXXI*) and they were tested against the following six microorganisms: *Streptococcus beta haemolyticus* C I. 4/49, *Streptococcus faecalis* 16/66, *Staphylococcus pyogenes aureus* (Oxford) Mau 1/45, *Escherichia coli* 5/49, *Proteus vulgaris* PrO 2/35 and *Pseudomonas aeruginosa* 26/56. For comparison substituted 3,4-secolupane acids were also included in the tested series, which were prepared in our preceding studies (*XXXIII*–*XXXVI* (ref.[1]) and *V*, *XXXII* (ref.[3])), four acids from the series of 3,4-seco-19β,28-epoxy-18α-oleanane (*XXXVII*–*XXXIX* (ref.[4]) and *XL* (ref.[10])) and the steroidal antibiotic – fusidic acid (*XLI*) – as a standard substance. None of the substances tested showed any activity against *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa* in concentrations lower than 50 μg/ml. The minimum inhibitory concentrations

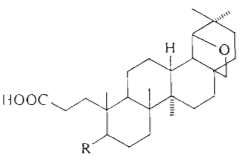
TABLE I

Important ions, *m/z* (%), in the mass spectra of 3,4-secolupane derivatives. Relative abundance of the ions is referred to the most abundant ion in the region of *m/z* higher than 250

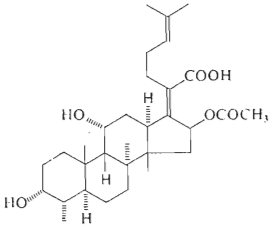
Compound	$M^{+\cdot}$	$[M-a]^{+\cdot}$	$[M-b]^+$	$[M-a-b]^+$	$[M-c]^+$	$[M-a-c]^+$	d^+
IX	474 (3)	428 (100)	401 (18)	355 (5)	—	—	261 (10)
X	502 (13)	442 (100)	415 (30)	355 (10)	—	—	275 (7)
XI	488 (3)	442 (100)	401 (41)	355 (11)	—	—	261 (11)
XII	488 (48)	428 (100)	415 (49)	355 (28)	—	—	275 (23)
XVI	530 (3)	484 (15)	457 (6)	411 (15)	419 (42)	373 (100)	289 (56)
XXVII	502 (1)	456 (23)	429 (3)	383 (11)	391 (37)	345 (100)	261 (19)
XXVIII	516 (1)	470 (13)	429 (5)	383 (10)	405 (28)	359 (100)	261 (10)
XXIX	544 (4)	484 (5)	457 (3)	397 (5)	419 (48)	359 (100)	275 (13)
XXX	530 (4)	470 (10)	457 (5)	397 (9)	405 (100)	345 (83)	275 (16)
XXXI	516 (9)	456 (22)	443 (11)	383 (26)	405 (100)	345 (74)	275 (39)



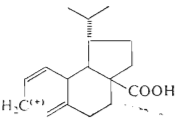
XXXII, $R^1 = CH_2{=}\overset{|}{C}{-}CH_3$, $R^2 = CH_3$
XXXIII, $R^1 = CH_3{-}\overset{|}{C}H{-}CH_2OH$, $R^2 = H$ (4*S*)
XXXIV, $R^1 = CH_3{-}\overset{|}{C}H{-}CH_2OH$, $R^2 = H$ (4*R*)
XXXV, $R^1 = (CH_3)_2\overset{|}{C}{-}OH$, $R^2 = CH_3$
XXXVI, $R^1 = (CH_3)_2\overset{|}{C}{-}OH$, $R^2 = H$



XXXVII, $R = CH_2{=}\overset{|}{C}{-}COOH$
XXXVIII, $R = CH_2{=}\overset{|}{C}{-}CH_2OH$
XXXIX, $R = CH_3{-}\overset{|}{C}H{-}CH_2OH$ (4*R*)
XL, $R = (CH_3)_2\overset{|}{C}{-}OH$



XLI



a'

against other microorganisms are summarized in Table II. The most active substance against *Staphylococcus aureus* is diacid *XXXVII*; its activity is, however, about a thousand times lower than the activity of fusidic acid (*XLI*). When comparing the effects of free di- and triacids and their esters a more general dependence between the esterification of some of the carboxyl groups and the antibacterial activity against *Staphylococcus aureus* could not be found. Important differences between the effect of the derivatives from the 18α-oleanane and lupane series also could not be found. The activity of the substances against *Streptococcus faecalis* is approximately the same as against *Staphylococcus aureus*. In all substances tested the highest activity was observed against *Streptococcus beta haemolyticus*. Here the activity of 3,4-seco-acids is comparable to that of fusidic acid (*XLI*); the most active was diacid *V*. From Table II it is evident that in the case of di- and triacids the esterification of some of the carboxyl groups is accompanied by a mild increase in activity.

EXPERIMENTAL

The melting points were determined on a Kofler block. Optical rotations were measured on a ETL-NPL (Bendix-Ericsson) polarimeter, with a $\pm 2°$ accuracy, in chloroform solution ($c = 0{\cdot}3-0{\cdot}8$) unless stated otherwise. The infrared spectra were measured on UR 10 and UR 20 (Zeiss, Jena) spectrophotometers in chloroform, unless otherwise stated. The ultraviolet spectra were measured with a Unicam SP 700 spectrophotometer in cyclohexane. The ^{1}H NMR spectrum was measured in deuteriochloroform solution on a Varian HA-100 instrument, using tetramethylsilane as internal reference. The mass spectra were measured on JEOL JMS-100 and Varian MAT 311 instruments. For thin-layer chromatography silica gel G according to Stahl (Merck) was used, while for column chromatography Silpearl silica gel (Kavalier, Votice) and neutral alumina of act. II (Reanal) were employed. Under the "conventional working up" the following procedure is understood: washing of the extract with water, a sodium or potassium hydrogen carbonate solution, and water, drying over sodium sulfate and evaporation of the solvent under reduced pressure. For the washing of the organic solutions dilute hydrochloric acid (1 : 4) was used. Unless stated otherwise the methyl esters were prepared from the acids with ethereal diazomethane solution. The identity of the substances was checked by comparison of their infrared spectra, chromatography on thin layers and mixture melting point determination. The analytical

TABLE II

Minimum inhibitory concentrations (μg/ml) of 3,4-secoacids

Compound	*Streptococcus beta haemolyticus*	*Streptococcus faecalis*	*Staphylococcus pyogenes aureus*
V	<0·1	50	25
XVI	3·12	>50	>50
XXIII	1·56	25	>50
XXIV	3·12	>50	>50
XXVI	0·8	50	>50
XXVII	1·56	>50	25
XXVIII	0·8	>50	>50
XXX	0·4	>50	>50
XXXI	1·56	25	25
XXXII	1·56	>50	>50
XXXIII	1·56	>50	50
XXXIV	6·25	>50	>50
XXXV	0·8	12·5	50
XXXVI	6·25	>50	>50
XXXVII	1·56	12·5	12·5
XXXVIII	6·25	25	25
XXXIX	1·56	25	25
XL	3·12	50	50
XLI	0·4	1·56	0·02

samples were dried under reduced pressure over phosphorus pentoxide for 8 h; higher melting substances at 100°C, low melting and amorphous at 20°C.

3,4-Seco-4(23),20(29)-lupadiene-3,28-dioic Acid (*V*)

Chromium trioxide (6 g), followed by sulfuric acid (0·5 ml) was added dropwise and under stirring to a solution of 6 g of betulinic acid (*I*) in dimethylformamide (110 ml) and the mixture was allowed to stand at room temperature for 24 h. After dilution with water the precipitated product was extracted with chloroform. The chloroform extract was washed repeatedly with a saturated ammonium sulfate solution, dried over sodium sulfate and chloroform was distilled off. The residue, betulonic acid (*II*), was dissolved in pyridine (180 ml) and hydroxylamine hydrochloride (6 g) was added to it. The mixture was heated at 100°C for 1 h, diluted with water, the precipitated material was filtered off under suction and washed with water. The product *III*, m.p. 222—225°C (literature[3] gives m.p. 228—229°C) was dried at 90°C. Oxime *III* was dissolved in pyridine (125 ml), *p*-toluenesulfonyl chloride (12·5 g) was added to it and the mixture was refluxed for 6 h. Water was added and the mixture extracted with chloroform. The chloroform layer was washed with dilute hydrochloric acid and water, dried over sodium sulfate and the solvent was evaporated under reduced pressure. According to thin-layer chromatography the residue contained nitrile-acid *IV* and a small amount of corresponding anhydride[3]. Ethylene glycol (295 ml) was added to it, followed by sodium hydroxide (29·5 g). The mixture was refluxed for 7 h, acidified with dilute hydrochloric acid and the separated diacid *V* was extracted with ether. The ethereal layer was washed with water and the diacid *V* was purified from the neutral fractions by extraction into 5% sodium carbonate solution. The extract was acidified with dilute hydrochloric acid and the precipitated diacid *V* was extracted with ether. The ethereal extract was washed with water, dried over sodium sulfate and evaporated. The residue (4 g) was dissolved in ethanol and filtered through a layer of active charcoal covering a layer of silica gel. After the evaporation of ethanol 3·1 g of diacid *V* were obtained, m.p. 239—241°C (ether–light petroleum), with a change of modification at 188—200°C (ref.[3] gives 243—246°C).

3-Methyl Ester of 3,4-Seco-4(23),20(29)-lupadiene-3,28-dioic Acid (*VII*)

Dowex 50 WX2 in H^+ form (50 mg) was added to a solution of diacid *V* (53 mg) in methanol (4 ml) and the mixture was refluxed for 8 h. Dowex was filtered off and the filtrate concentrated. The residual product was purified by thin-layer chromatography (20 × 20 cm plates) on silica gel in light petroleum–ether (7: 3). Methyl ester *VII* (40 mg) crystallized from an ether–light petroleum mixture, m.p. 216·5—218°C, $[\alpha]_D$ +23°. IR spectrum: 2 600—3 250, 1 693 (COOH), 3 079, 1 639, 890 (C=CH_2), 1 729, 1 436, 1 163 cm^{-1} ($COOCH_3$). For $C_{31}H_{48}O_4$ (484·7) calculated: 76·81% C, 9·98% H; found: 76·95% C, 10·08% H.

3-Methyl Ester of 3,4-Secolupane-3,28-dioic Acid (*XI*)

Diacid *IX* (see lit.[3], 100 mg) was esterified with methanol as in the preparation of ester *VII*. Yield, 75 mg of ester *XI*, m.p. 256—257°C (chloroform–methanol), $[\alpha]_D$ −21°. IR spectrum: 2 400—3 400, 1 699 (COOH), 1 735, 1 442, 1 172 cm^{-1} ($COOCH_3$). Mass spectrum, *m/z* (%): M^+ 488 (3), 470 (3), 456 (3), 443 ($C_{30}H_{51}O_2$; 38), 442 ($C_{30}H_{50}O_2$; 100), 402 (15), 401 (41), 399 (24), 355 (11), 263 (17), 261 (11). For $C_{31}H_{52}O_4$ (488·7) calculated: 76·18% C, 10·72% H; found: 76·40% C, 10·94% H.

28-Methyl Ester of 3,4-Secolupane-3,28-dioic Acid (*XII*)

A solution of dimethyl ester *X* (see lit.[3]; 100 mg) in benzene (7 ml) was mixed with 3,5 ml of 1M-KOH in methanol. The mixture was refluxed for 2 h, then shaken with dilute hydrochloric acid and extracted with ether. The ethereal extract was washed with water until neutral, dried over sodium sulfate and evaporated. The residue was crystallized from methanol. Yield, 80 mg of ester *XII*, m.p. 187–188°C, $[\alpha]_D$ −18°. IR spectrum: 2 400–3 400, 1 715 (COOH), 1 715, 1 440, 1 167 cm^{-1} ($COOCH_3$). Mass spectrum, *m/z* (%): M^+ 488 ($C_{31}H_{52}O_4$; 48), 470 (10), 456 (13), 445 (8), 429 ($C_{29}H_{49}O_2$; 89), 428 ($C_{29}H_{48}O_2$; 100), 416 (18), 415 ($C_{28}H_{47}O_2$; 49), 385 (36), 355 (28), 275 (23). For $C_{31}H_{52}O_4$ (488·7) calculated: 76·18% C, 10·72% H; found: 76·04% C, 10·74% H.

Dimethyl Ester of 23,29-Dioxo-3,4-seco-4(24),20(30)-lupadiene-3,28-dioic Acid (*XIII*)

Selenium dioxide (6 g) was added to a solution of dimethyl ester *VI* (see lit[3]; 2·2 g) in acetic acid (100 ml) and the mixture was refluxed for 6 h. It was filtered through a double layer of charcoal and silica gel (lower bed) and the layer was washed with acetic acid. The filtrate was evaporated and the residue partitioned between ether and water. The ethereal extract was worked up in the conventional manner. The product (1·7 g) was chromatographed on alumina (100 g). Elution of the mixture with light petroleum–ether (1 : 1) gave 0·23 g of dialdehyde *XIII*, m.p. 158·5 to 160°C (ether–light petroleum), with crystal modification change at 105–115°C, $[\alpha]_D$ −17°. IR spectrum: 3 098, 1 618, 952 (C=CH_2), 2 710, 1 692 (CHO), 1 723, 1 439, 1 161 cm^{-1} ($COOCH_3$). UV spectrum: λ_{max} 225 nm ($\log \varepsilon = 4{\cdot}10$). For $C_{32}H_{46}O_6$ (526·7) calculated: 72·97% C, 8·80% H; found: 73·05% C, 8·97% H.

3,28-Dimethyl Ester, 23,29-Dinitrile of 3,4-Seco-4(24),20(30)-lupadiene-3,23,28,29-tetraoic Acid (*XV*)

Hydroxylamine hydrochloride (350 mg) was added to a solution of dialdehyde *XIII* (130 mg) in pyridine (10 ml) and the mixture was heated at 100°C for 7 h. After dilution with water the precipitate formed was extracted with ether. The ethereal extract was washed with water, dilute hydrochloric acid and further worked up in the conventional manner. The crude oxime *XIV* was characterized only by IR spectrum: 3 602, 3 380 (OH), 3 105, 1 623, 1 599, 955, 918 (CH_2=C–CH=NOH), 1 721, 1 439, 1 161 cm^{-1} ($COOCH_3$). Oxime *XIV* was dissolved in acetic anhydride (20 ml) and the mixture refluxed for 5 h. After dilution with water the precipitated product was extracted with ether. The ethereal extract was worked up in the conventional manner. Dinitrile *XV* was chromatographed on a column of alumina (15 g). Elution with light petroleum–ether (7 : 3 and 6 : 4) gave 80 mg of a chromatographically pure dinitrile *XV*, m.p. 160–161°C (ether–methanol), $[\alpha]_D$ +5°. IR spectrum: 2 218 (CN), 3 110, 1 620, 940 (C=CH_2), 1 726, 1 440, 1 162 cm^{-1} ($COOCH_3$). UV spectrum: λ_{max} 210 nm ($\log \varepsilon = 4{\cdot}11$). For $C_{32}H_{44}N_2O_4$ (520·7) calculated: 73·81% C, 8·52% H; found: 74·19% C, 8·35% H.

3,4-Seco-4(24),20(30)-lupadiene-3,23,28,29-tetraoic Acid (*XVI*)

Dinitrile *XV* (40 mg) was hydrolysed with 10% sodium hydroxide in ethylene glycol, as in the preparation of diacid *V*. After filtration through a layer of charcoal 30 mg of tetraacid *XVI* were obtained, m.p. 305–307°C (decomposition; ether–light petroleum), $[\alpha]_D$ −9° (acetone). IR spectrum (KBr): 2 400–3 700, 1 698 (COOH), 1 619, 942 cm^{-1} (C=CH_2). Mass spectrum, *m/z* (%): M^+ 530 (3), 512 (17), 494 (12), 484 (15), 466 (21), 458 (9), 457 (6), 448 (11), 419 (42), 413 (20), 411 (15), 401 (42), 395 (20), 383 (27), 373 (100), 365 (15), 355 (4), 337 (25), 289 (56).

28-Methyl Ester, 3-Nitrile of 23-Oxo-3,4-seco-4(24)-lupene-3,28-dioic Acid (*XVIII*)

Selenium dioxide (6 g) was added to a solution of nitrile *XVII* (see lit.[3]; 5 g) in acetic acid (250 ml) and the mixture was refluxed for 5 h. After working up as in the case of dialdehyde *XIII* the residue was dissolved in a mixture of light petroleum and benzene (1 : 1) and chromatographed on alumina (200 g). Elution with benzene and a benzene–ether mixture (9 : 1) gave aldehyde *XVIII* (2·25 g), which was crystallized from light petroleum, m.p. 130–136°C (change of crystal modification at 97–99°C), $[\alpha]_D$ −35°. IR spectrum: 3 094, 1 619, 955 (C=CH_2), inflexion at 2 827, 2 710, 1 690 (CHO), 2 246 (CN), 1 719, 1 437, 1 163 cm^{-1} ($COOCH_3$). UV spectrum: λ_{max} 216 nm ($\log \varepsilon = 3{\cdot}86$). For $C_{31}H_{47}NO_3$ (481·7) calculated: 77·29% C, 9·84% H; found: 77·57% C, 10·08% H.

28-Methyl Ester, 3 Nitrile of 23-Hydroxy-3,4-seco-4(24)-lupene-3,28-dioic Acid (*XIX*)

Sodium boro-hydride (0·5 g) was added to a solution of aldehyde *XVIII* (0·54 g) in benzene (40 ml) and methanol (20 ml) and allowed to stand at room temperature for 3 h. After dilution with water, the mixture was acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was worked up in the conventional manner. The residue (0·55 g) was chromatographed on silica gel (50 g). Elution with a light petroleum–ether (6 : 4) mixture afforded 0·34 g of hydroxy nitrile *XIX*, m.p. 111–113°C (ether–light petroleum), $[\alpha]_D$ −13°. IR spectrum: 3 619 (OH), 3 090, 1 642, 907 (C=CH_2), 2 246 (CN), 1 719, 1 432, 1 159 ($COOCH_3$), 1 020 cm^{-1} (C—O). For $C_{31}H_{49}NO_3$ (483·7) calculated: 76·97% C, 10·21% H; found: 76·70% C, 10·56% H.

Further elution with ether gave 100 mg of hydroxy nitrile *XXII*, which was identical with the preparation described in ref.[1]. M.p. 166–167°C (ether–light petroleum), $[\alpha]_D$ −27°. IR spectrum: 3 634 (OH), 2 247 (CN), 1 719, 1 435, 1 159 ($COOCH_3$), 1 012 cm^{-1} (C—O).

28-Methyl Ester, 3,23-Dinitrile of 3,4-Seco-4(24)-lupene-3,23,28-trioic Acid (*XXI*)

A solution of aldehyde *XVIII* (0·9 g) in pyridine (50 ml) was mixed with hydroxylamine hydrochloride (0·9 g) and heated at 100°C for 5 h. The mixture was worked up as in the case of oxime *XIV* (see the preparation of dinitrile *XV*). The crude oxime *XX* was characterized only by its IR spectrum: 3 594, 3 395 (OH), 3 105, 1 620, 1 597, 952 (CH_2—C—CH=NOH), 2 241 (CN), 1 719, 1 436, 1 162 cm^{-1} ($COOCH_3$). The crude oxime *XX* was dissolved in acetic anhydride (70 ml), the solution was refluxed for 3 h and the mixture worked up as in the case of dinitrile *XV*. The product (0·8 g) was chromatographed on alumina (70 g). Dinitrile *XXI* (0·6 g) was obtained on elution with light petroleum–benzene (1 : 1) and benzene. M.p. 182–183°C (ether–light petroleum), with crystal modification change at 74–80°C (if the sample is put into a Kofler block at 90°C, it melts immediately and then crystallizes; m.p. is then 183–184°C), $[\alpha]_D$ −10°. IR spectrum: 3 034, 1 612, 938 (C=CH_2), 2 248, 2 222 (CN), 1 718, 1 432, 1 160 cm^{-1} ($COOCH_3$) UV spectrum: λ_{max} 204 nm ($\log \varepsilon = 3{\cdot}83$). For $C_{31}H_{46}N_2O_2$ (478·7) calculated: 77·78% C, 9·69% H; found: 78·07% C, 9·63% H.

23-Hydroxy-3,4-seco-4(24)-lupene-3,28-dioic Acid (*XXIII*)

Hydroxy nitrile *XIX* (310 mg) was hydrolysed with sodium hydroxide in ethylene glycol, the same as in the preparation of diacid *V*. After filtration through charcoal 260 mg of diacid *XXIII* were obtained, m.p. 254–257°C (ether–light petroleum), $[\alpha]_D$ −18° (acetone). IR spectrum (KBr): 3 440 (OH), 2 400–3 650, inflexion 1 699, 1 704 (COOH), 3 090, 1 645, 905 (C=CH_2), 1 048 cm^{-1}

(C—O). For $C_{30}H_{48}O_5$ (488·7) calculated: 73·73% C, 9·90% H; found: 73·50% C, 9·67% H.

Dimethyl ester *XXV* was purified by preparative chromatography on silica gel thin layers (20 × 20 cm) in light petroleum–acetone (8 : 2). M.p. 141—143°C (light petroleum), $[\alpha]_D$ −20°. IR spectrum: 3 624 (OH), 3 094, 1 648, 903 (C=CH_2), 1 732, 1 443, 1 177, 1 163 cm^{-1} ($COOCH_3$) For $C_{32}H_{52}O_5$ (516·7) calculated: 74·37% C, 10·14% H; found: 74·61% C, 10·36% H.

23-Acetoxy-3,4-seco-4(24)-lupene-3,28-dioic Acid (*XXIV*)

Acetic anhydride (1 ml) was added to a solution of diacid *XXIII* (60 mg) in pyridine (2 ml) and the mixture was allowed to stand at room temperature for 5 days. After pouring on ice and standing for a few hours the separated product was extracted with ether. The ethereal phase was washed with water, dilute hydrochloric acid, again with water and then dried over sodium sulfate. After filtration ether was distilled off. Yield, 60 mg of an oily product in the IR spectrum of which absorption bands were present corresponding to the free carboxyl group (2 400—3 400, 1 705 cm^{-1}) and also to the mixed anhydride with acetic acid (1 808 cm^{-1}). A solution of the product (60 mg) in dioxane (40 ml) was heated to its boiling point, 20 ml of water were added gradually under refluxing, which was continued for another 4 h. After dilution with a saturated ammonium sulfate solution the precipitated product was extracted with ether. The extract was washed with water, dried over sodium sulfate and evaporated in a vacuum. The product was purified by chromatography on a silica gel column (5 g). Elution with light petroleum–ether (7 : 3) gave acetate *XXIV* (40 mg), m.p. 220—222·5°C (light petroleum with a trace of ether), $[\alpha]_D$ −10°. IR spectrum: 3 523, 2 400—3 350, 1 703 (COOH), 1 734, 1 240 ($OCOCH_3$), 1 647, 914 cm^{-1} (C=CH_2). For $C_{32}H_{50}O_6$ (530·7) calculated: 72·41% C, 9·50% H; found: 72·65% C, 9·66% H.

28-Methyl Ester of 23-Hydroxy-3,4-seco-4(24)-lupene-3,28-dioic Acid (*XXVI*)

A 1M-KOH solution (0·3 ml) in methanol was added to a solution of dimethyl ester *XXV* (70 mg) in benzene (10 ml) and the mixture was refluxed for 4 h and then worked up as in the preparation of ester *XII*. The residue (70 mg) was chromatographed on a silica gel column (5 g) with light petroleum–ether (1 : 1), which eluted 60 mg of ester *XXVI*, m.p. 128—131°C (ether–light petroleum), $[\alpha]_D$ −18°. IR spectrum: 3 614, 3 524 (OH), 2 400—3 400, 1 716 (COOH), 1 716, 1 438, 1 160 ($COOCH_3$), 1 640, 922 cm^{-1} (C=CH_2). For $C_{31}H_{50}O_5$ (502·7) calculated: 74·06% C, 10·03% H; found: 74·07% C, 10·42% H.

3,4-Seco-4(24)-lupene-3,23,28-trioic Acid (*XXVII*)

Dinitrile *XXI* (480 mg) was hydrolysed as in the preparation of diacid *V*. Yield, 350 mg of triacid *XXVII*, m.p. 295—297°C (ether–light petroleum), $[\alpha]_D$ −19° (acetone). IR spectrum (KBr): 2 380—3 700, 1 710 (COOH), 1 625, 952 cm^{-1} (C=CH_2). UV spectrum: λ_{max} 196 nm. Mass spectrum, *m/z* (%): M^+ 502 (1), 484 (7), 456 (23), 438 (5), 430 (3), 429 (3), 413 (12), 397 (13), 391 ($C_{24}H_{39}O_4$; 37), 383 (11), 373 (8), 355 (9), 345 ($C_{23}H_3$-O_2; 100), 327 (14), 261 (19). For $C_{30}H_{46}O_6$ (502·7) calculated: 71·68% C, 9·27% H; found: 71·77% C, 9·33% H.

Trimethyl ester *XXXIX* was prepared on reacting triacid *XXVII* (100 mg) with a dilute ethereal diazomethane solution for 1 h (the reaction was monitored by thin-layer chromatography, in order to prevent the formation of the pyrazoline derivative in the side chain at $C_{(5)}$). The product was chromatographed on thin layers of silica gel (20 × 20 cm plates) in light petroleum–ether (7 : 3). Yield, 70 mg of amorphous trimethyl ester *XXIX*; $[\alpha]_D$ −9°. IR spectrum: inflexion 1 725, 1 721, inflexion 1 714, 1 437, 1 163 ($COOCH_3$), 1 620, 946 cm^{-1} (C=CH_2). ^1H NMR spectrum (in ppm, δ-scale): 0·76 s, 0·96 s and 0·98 s (3 × CH_3), 0·76 d J = 6·6 Hz and 0·85 d

J = 6·9 Hz ($(CH_3)_2CH$), 2·00–2·83 unresolved multiplet, 3·64 s, 3·65 s and 3·71 s (3 × × $COOCH_3$), 5·48 bs and 6·21 bs ($C{=}CH_2$). Mass spectrum, *m/z* (%): M^+ 544 (4), 512 (7), 484 ($C_{31}H_{48}O_4$; 5), 458 ($C_{29}H_{46}O_4$; 5), 457 (3), 419 ($C_{26}H_{43}O_4$; 48) 397 (5), 359 ($C_{24}H_{39}O_2$; 100), 327 (6), 275 (13). For $C_{33}H_{52}O_6$ (544·7) calculated: 72·75% C, 9·62% H; found: 72·57% C, 9·70% H.

3-Methyl Ester of 3,4-Seco-4(24)-lupene-3,23,28-trioic Acid (*XXVIII*)

Triacid *XXVII* (60 mg) was partially esterified with methanol in the same manner as diacid *V* (see the preparation of ester *VII*). The product was purified by preparative chromatography on silica gel thin layers (20 × 20 cm) in benzene–acetone (3 : 1). Yield, 35 mg of ester *XXVIII* which was crystallized from ether. M.p. 244–248°C, with crystal modification change at 234–240°C, $[\alpha]_D$ −13°. IR spectrum (KBr): 2 400–3 640, 1 699 (COOH), 1 744, inflexion 1 441, 1177 ($COOCH_3$), 1 623, 954 cm^{-1} ($C{=}CH_2$). Mass spectrum, *m/z* (%): M^+ 516 (1), 498 (5), 470 (13), 430 (5), 429 (5), 427 (5), 405 ($C_{25}H_{41}O_4$; 28), 383 (10), 359 ($C_{24}H_{39}O_2$; 100), 327 (5), 261 (10). For $C_{31}H_{48}O_6$ (516·7) calculated: 72·06% C, 9·36% H; found: 72·15% C, 9·66% H.

23,28-Dimethyl Ester of 3,4-Seco-4(24)-lupene-3,23,28-trioic Acid (*XXX*)

1M-KOH solution in methanol (9 ml) was added to a solution of trimethyl ester *XXIX* (65 mg) in benzene (10 ml) and the mixture was refluxed for 1 h. After working up as in the preparation of ester *XII* the residue was dissolved in a mixture of light petroleum and ether (8 : 2) and chromatographed on a silica gel column (5 g). Ester *XXX* (55 mg) was obtained by elution with light petroleum–ether mixture (7 : 3). M.p. 171–174°C (ether–pentane), $[\alpha]_D$ −7°. IR spectrum: 3 534, 2 400–3 400, inflexion 1 706 (COOH), 1 714, 1 439, 1 161 ($COOCH_3$), 1 621, 948 cm^{-1} ($C{=}CH_2$). Mass spectrum, *m/z* (%): M^+ 530 (4), 498 (6), 470 (10), 458 (4), 457 (5), 405 ($C_{25}H_{41}O_4$; 100), 397 ($C_{27}H_{41}O_2$; 9), 345 ($C_{23}H_{37}O_2$; 83), 327 (8), 275 ($C_{18}H_{27}O_2$; 16). For $C_{32}H_{50}O_6$(530·7) calculated: 72·41% C, 9·50% H; found: 71·92% C, 9·62% H.

28-Methyl Ester of 3,4-Seco-4(24)-lupene-3,23,28-trioic Acid (*XXXI*)

Water (10 ml) and sodium hydroxide (400 mg) were added to a solution of trimethyl ester *XXIX* (100 mg) in dioxane (10 ml) and the mixture was refluxed for 6 h and worked up as in the case of diacid *V*. Yield, 80 mg of ester *XXXI*, m.p. 270–271°C (ether–light petrooleum), $[\alpha]_D$ +5°. IR spectrum: 2 400–3 400, inflexion 1 697 (COOH), 1 712, 1 436, 1 162 ($COOCH_2$), 1 620, 958 cm^{-1} ($C{=}CH_2$). Mass spectrum, *m/z* (%): M^+ 516 (9), 498 (17), 470 (9), 457 (30), 456 (22), 444 (13), 443 (11), 439 (26), 438 (17), 413 (15), 405 (100), 397 (22), 395 (26), 383 (26), 345 (74), 275 (39). For $C_{31}H_{48}O_6$ (516·7) calculated: 72·06% C, 9·36% H; found: 72·51% C, 9·23% H.

Bioassay Procedure

The minimum inhibition concentrations were determined by dilution method at pH 6·0. The tested substance was dissolved in 50% dimethyl sulfoxide and diluted with the nutrient broth No 2 (IMUNA) to the following concentrations (μg/ml): 50, 25, 12·5, 6·25, 3·12, 1·56, 0·8, 0·4, 0·2 and 0·1. 0·1 ml of a bacterial culture of the mentioned strains, of 10^6 bact/ml concentration, was added to the diluted substance in 2 ml of the mentioned broth. In addition to this 0·5 ml of 50% dimethyl sulfoxide were added to each strain in a corresponding amount of the nutrient broth No 2, in order to determine a possible inhibition due to the solvent. The inhibition of growth was determined in the nutrient broth after 24 h of cultivation at 37°C.

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