Cytotoxic Saponins from the Chinese Herbal Drug Yunnan Bai Yao

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Received August 14, 1978, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706.* Accepted for publication January 19, 1979.

Abstract □ Two cytotoxic saponins were isolated from the acetone extracts of the herbal preparation "Yunnan Bai Yao" by column chromatography. They were identified as 3-{[α-L-rhamnopyranosyl(1_{Rha} → 2_{Glu})]-[α-L-arabinofuranosyl(1_{Ara} → 4_{Glu})]-β-D-glucopyranosyl}-25(*R*)-spirost-5-en-3β-ol (I) and 3-{[α-L-rhamnopyranosyl(1_{Rha} → 4_{Rha})-α-L-rhamnopyranosyl(1_{Rha} → 4_{Glu})]-[α-L-rhamnopyranosyl(1_{Rha} → 2_{Glu})]-β-D-glucopyranosyl}-25(*R*)-spirost-5-en-3β-ol (VI). Both saponins exhibited significant cytotoxicity in the standard P-388, L-1210, and 9KB tissue culture screens.

Keyphrases □ Folk medicine—Yunnan Bai Yao, Chinese herbal, cytotoxic activity of isolated saponins □ Yunnan Bai Yao—analysis, cytotoxic saponins, Chinese herbal □ Antineoplastic agents, potential—Yunnan Bai Yao, Chinese herbal, cytotoxic activity of isolated saponins □ Hemostatic agents—Yunnan Bai Yao, cytotoxic activity of isolated saponins, Chinese herbal

The Chinese herbal preparation "Yunnan Bai Yao" was formulated with indigenous medicinal plants about 50 years ago in the Yunnan province. This powdered herbal mixture has been used primarily as a hemostatic agent and gained wide acceptance during the Sino-Japanese War (1937–1945) (1). Claims regarding its efficacy in promoting wound healing and relieving pain also have been made.

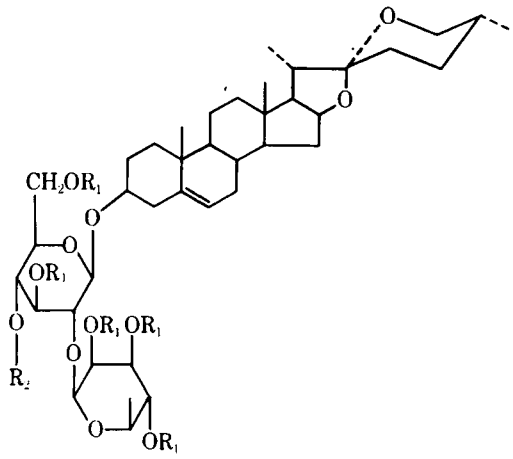
In recent years, rumors have circulated among the overseas Chinese that Yunnan Bai Yao could be used in cancer therapy (2). Since extracts of Yunnan Bai Yao exhibited a confirmed level of activity (3–5) in the National Cancer Institute's P-388, L-1210, and 9KB tissue culture screens, the cytotoxic components of this herbal preparation were of interest.

EXPERIMENTAL

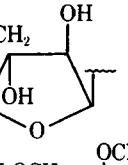
Melting points were determined in open capillary tubes[1] and are uncorrected. Optical rotations were measured with a polarimeter[2]. NMR spectra[3] were determined in deuterochloroform containing tetramethylsilane as the internal standard. Mass spectra[4] were recorded at an ionizing voltage of 70 ev. Descending paper chromatography was carried out on Whatman No. 1 paper, which was developed for 48 hr in *n*-butanol–ethanol–water (4:1:5, upper phase). Sugars were visualized with alkaline silver nitrate spray.

Silica gel[5] was used for column chromatography. A gas chromatograph[6], equipped with a 3.66-m column (2.16 mm i.d.) of 5% SE-30 on Chromosorb and operated at 190°, was used for the separation of trimethylsilyl ethers (6) of the sugars. The carrier nitrogen gas flow was 40 ml/min. For the separation of methylated and partially methylated methyl glycosides, a 1.83-m column of 15% butanediol succinate (7) on Chromosorb W-AW (2.16 mm i.d.) at 175° was used instead. Monoglucoside and maltoside of 25(*R*)-spirost-5-en-3β-ol (II) were prepared by known methods (8).

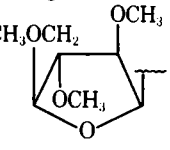
Herbal Preparation of Yunnan Bai Yao—Commercially[7] available



I: R_1 = H, R_2 = $HOCH_2$



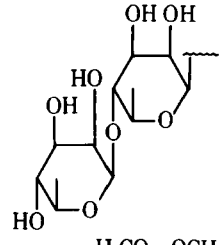
III: R_1 = CH_3, R_2 = CH_3OCH_2



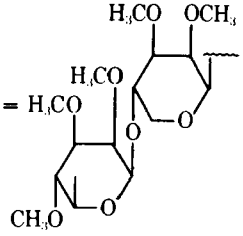
IV: R_1 = R_2 = H

V: R_1 = R_2 = CH_3

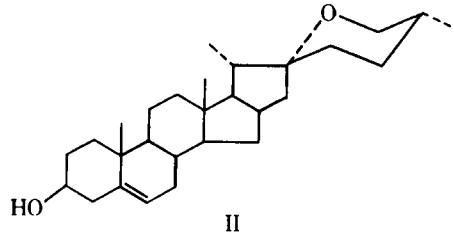
VI: R_1 = H, R_2 =



VII: R_1 = CH_3, R_2 =



VIII: R_1 = CH_3, R_2 = H



II

[1] Thomas-Hoover apparatus.
[2] Perkin-Elmer model 241.
[3] Varian model EM-390.
[4] AEI MS-9.
[5] MN-Kieselgel, 70-270 mesh ASTM.
[6] Varian Aerograph model 1860-1.
[7] Manufactured in Kuming, Yunnan, China, and purchased in Hong Kong.

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Yunnan Bai Yao was used. The preparation and its chemical constituents were described previously (2).

Fractionation and Isolation of I and VI (Scheme I)—A total of 1.3 kg of Yunnan Bai Yao was extracted successively with 10 liters of benzene for 24 hr, followed by 10 liters of acetone for 48 hr. After solvent evaporation *in vacuo*, 31 g of cytotoxic residue was obtained from the acetone extract. This residue (31 g) was chromatographed on a silica gel (2-kg) column (72 × 9.4 cm). The column was eluted with chloroform–methanol–water (65:25:10), and 900-ml fractions were collected. After solvent evaporation *in vacuo* from fractions 11–16, 5.15 g of a white solid was obtained. Crystallization from 95% ethanol afforded pure 3-{[α-L-rhamnopyranosyl(1_{Rha} → 2_{Glu})]-[α-L-arabinofuranosyl(1_{Ara} → 4_{Glu})]-β-D-glucopyranosyl}-25(*R*)-spirost-5-en-3β-ol (I), mp 242–248°; $[\alpha]_D^{25}$ −118.6° (c, 0.51, methanol); IR (KBr): 3550–3110, 981, 912, 894, and 862 cm^{-1}.

Anal.—Calc. for $C_{44}H_{70}O_{16}\cdot 2H_2O$: C, 59.32; H, 8.31. Found: C, 59.55; H, 8.39.

Fractions 24–33 were combined to afford 2.57 g of 3-{[α-L-rhamnopyranosyl(1_{Rha} → 4_{Rha})-α-L-rhamnopyranosyl(1_{Rha} → 4_{Glu})]-[α-L-rhamnopyranosyl(1_{Rha} → 2_{Glu})]-β-D-glucopyranosyl}-25(*R*)-spirost-5-en-3β-ol (VI), mp 203–210°; $[\alpha]_D^{25}$ −110° (c, 0.50, 95% ethanol); IR (KBr): 3600–3100, 976, 908, 890, and 860 cm^{-1}.

Anal.—Calc. for $C_{51}H_{82}O_{20}\cdot 4H_2O$: C, 56.35; H, 8.28. Found: C, 56.50; H, 8.42.

Hydrolysis of I—The saponin I (100 mg) was heated with 2 *N* H_2SO_4 (4 ml) and dioxane (2 ml) at 100° for 10 hr. It was then diluted with 75 ml of water, and the mixture was extracted with ethyl acetate (4 × 75 ml). The organic layer was washed successively with sodium bicarbonate solution and water. After drying over sodium sulfate, the solvent was evaporated *in vacuo* to give 42 mg of II, mp 205–206°; $[\alpha]_D^{25}$ −115.7° (c, 0.53, chloroform).

The aqueous portion of the hydrolysate was neutralized with anion-exchange resins (carbonate form). After filtration and evaporation, a syrup resulted (52 mg); paper chromatography demonstrated spots corresponding to D-glucose, L-arabinose, and L-rhamnose. The identification of these sugars was further confirmed by reacting 5 mg each of the sugars with hexamethyldisilazane (0.4 ml) and trimethylsilyl chloride (0.2 ml) in pyridine (0.5 ml). After removal of the pyridine *in vacuo*, a hexane solution of the silylated sugars was subjected to GLC analyses.

Methylation of I (9)—Sodium hydride (500 mg, washed three times with hexane) in 40 ml of dimethyl sulfoxide was heated at 70° for 30 min under nitrogen with stirring. A solution of 500 mg of I in 25 ml of dimethyl sulfoxide was added. After stirring for 30 min, methyliodide (10 ml) was added. After stirring for 60 min, the reaction mixture was diluted with 400 ml of water and extracted with ether (4 × 100 ml).

The ethereal extract was washed with water (6 × 100 ml), dried over sodium sulfate, and evaporated to dryness to yield 504 mg of crude III. This procedure was repeated three times to obtain 497 mg of III, mp 159–161°; $[\alpha]_D^{25}$ − 92.5° (c, 1.03, chloroform).

Methanolysis of III (7)—After 350 mg of III was refluxed with methanolic hydrogen chloride (3 *N*, 75 ml) for 10 hr, the mixture was kept overnight at 25° and then evaporated *in vacuo*. Anhydrous methanol (100 ml) was added and again evaporated. After the addition of 75 ml of water, the aqueous layer was neutralized with anion-exchange resin (carbonate form) and then evaporated to yield 170 g of mixed methyl glycosides, which was subjected to GLC.

The methyl glycosides also were separated by column (2 × 23.5 cm) chromatography on silica gel (20 g). The column was eluted with a gradient system consisting of 200 ml of chloroform–methanol (99.5:0.5) in the mixing flask and 200 ml of chloroform–methanol (98:2) in the reservoir flask, and 10-ml fractions were collected. Fractions 12–16 afforded 41 mg of methyl 2,3,4-tri-*O*-methyl-L-rhamnopyranoside (III*a*). Fractions 18–29 gave 36 mg of methyl 2,3,5-tri-*O*-methyl-L-arabinofuranoside (III*b*). Fractions 37–48 yielded 42 mg of methyl 3,6-di-*O*-methyl-D-glucopyranoside (III*c*). These methyl glycosides were identified by mass spectral analyses (10).

Partial Hydrolysis of I—After 250 mg of I was heated with 10 ml of dioxane and 20 ml of 2 *N* H_2SO_4 at 75° for 1 hr, the mixture was diluted with 30 ml of water and neutralized with anion-exchange resin (carbonate form). The mixture was then filtered through a bed of Dowex 1 × 4 resin (carbonate form). The resin was washed successively with 200 ml of methanol followed by 200 ml of methanol–chloroform (1:1).

The filtrate and the washings were combined and evaporated *in vacuo* to yield 246 mg of residue, which was chromatographed on a silica gel (65 g) column (2.2 × 36.5 cm). The column was eluted with chloroform–methanol–water (65:25:10, organic phase), and 4-ml fractions were collected. Fractions 22–27 afforded 28 mg of 3-[β-D-glucopyranosyl]-25(*R*)-spirost-5-en-3β-ol (IV), mp 246–251°, $[\alpha]_D^{25}$ −87.9° (c, 0.52, methanol). Fractions 43–61 gave 120 mg of the prosapogenin (IV), mp 215° dec.; $[\alpha]_D^{25}$ −98.4° (c, 0.51, methanol).

Table I—Calculated and Experimental Molecular Rotations of Glycosides

Glycoside	$[M]_D$ Found	$[M]_D$ Calc.
3-[β-D-Glucopyranosyl]-25(*R*)-spirost-5-en-3β-ol	−572	−656
3-[α-L-Rhamnopyranosyl(1 → 2)-β-D-glucopyranosyl]-25(*R*)-spirost-5-en-3β-ol	−889	−853
3-{[α-L-Rhamnopyranosyl(1 → 2)]-[α-L-arabinofuranosyl(1 → 4)]-β-D-glucopyranosyl}-25(*R*)-spirost-5-en-3β-ol	−1034	−1064

Permethylation of IV—With the same procedure as described for the methylation of I, 120 mg of IV afforded 123 mg of crude V. Purification by column chromatography yielded 8.6 mg of pure V, mp 97–100°; $[\alpha]_D^{25}$ −101.5° (c, 0.8, chloroform).

Methanolysis of V—After 70 mg of V was treated with 30 ml of 2 *N* methanolic hydrogen chloride, the procedure used was the same as that described for III. A total of 30 mg of methyl glycoside was obtained, which was chromatographed on a silica gel (3-g) column (0.5 × 16.5 cm). The column was eluted with benzene–ethyl acetate (7:3), and 3-ml fractions were collected. Fractions 27–29 gave 8 mg of III*a*, and fractions 49–56 afforded 7.6 mg of methyl 3,4,6-tri-*O*-methyl-D-glucopyranoside (V*a*).

Hydrolysis of VI—Hydrolysis of VI (100 mg), carried out in the same manner as for I, yielded 39 mg of II. The sugars were identified by paper chromatography as L-rhamnose and D-glucose.

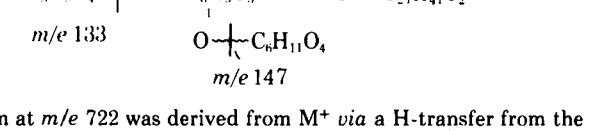
Methylation of VI—Following the methylation procedure for I, 750 mg of VI afforded 703 mg of VII, mp 116–118°; $[\alpha]_D^{25}$ −86° (c, 1.0, chloroform).

Methanolysis of VII—A mixture of 259 mg of methyl glycosides was obtained from methanolysis of 470 mg of VII. These glycosides were analyzed by GLC and further separated by column chromatography on silica gel with the same solvent system as described for the isolation of methyl glycosides from III. Fractions 13–19 gave 70 mg of III*a*, fractions 23–31 afforded 52 mg of methyl 2,3-di-*O*-methyl-L-rhamnopyranoside (VII*a*), and fractions 38–45 yielded 48 mg of III*c*.

Partial Hydrolysis of VII—After 190 mg of VII was refluxed with 0.5 *N* HCl in 40 ml of methanol for 3 hr, the mixture was evaporated, and 50 ml of water was added. The mixture was extracted with ethyl acetate (4 × 30 ml), and the ethyl acetate layer was washed with water, dried over sodium sulfate, and evaporated to yield 98 mg of residue. This residue was subjected to preparative TLC (20 × 20 cm, Kieselgel 60 F-256) using chloroform–acetone (85:15) as the solvent system. Compound VIII (R_f 0.46) was isolated and, upon methylation, yielded V.

RESULTS AND DISCUSSION

Acid hydrolysis of the saponin I afforded II, D-glucose, L-rhamnose, and L-arabinose. GLC analyses of the trimethylsilyl derivatives of these sugars revealed that they were present in a 1:1:1 ratio. The mass spectrum of I showed prominent peaks at *m/e* 133, 147, 414, 576, and 722. The peaks at *m/e* 133 and 147 correspond to arabinosyl and rhamnosyl ions, respectively, suggesting that these sugars are present in I as terminal units in branched chain fashion.



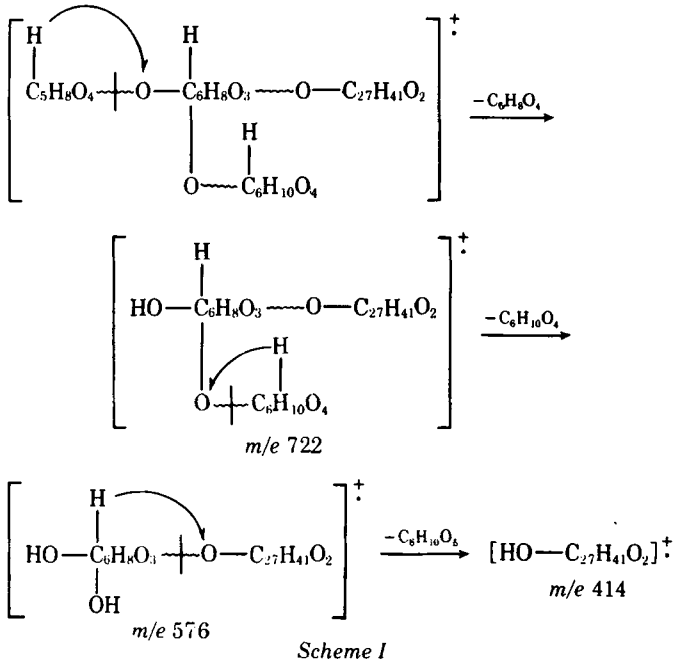
The ion at *m/e* 722 was derived from M^+ *via* a H-transfer from the arabinosyl group, accompanied by its concomitant elimination (Scheme I). In turn, the ion at *m/e* 576 originated from the ion at *m/e* 722 by a similar H-transfer from the terminal rhamnosyl group, accompanied by its elimination. The ion at *m/e* 414 was formed from the ion with *m/e* 576 by a H-transfer from the glucosyl group, followed by its elimination (11, 12), indicating that glucose was directly attached to the aglycone.

This proposed branched sugar sequence was further substantiated by the mass spectrum of the permethylated derivative (III), which gave prominent peaks at *m/e* 175 and 189, corresponding to terminal tri-*O*-methylarabinosyl and tri-*O*-methylrhamnosyl ions. The peak at *m/e* 435 may be rationalized by assuming that the terminal tri-*O*-methylrham-

Table II—Cytotoxic Activity of Natural and Synthetic Saponins [a]

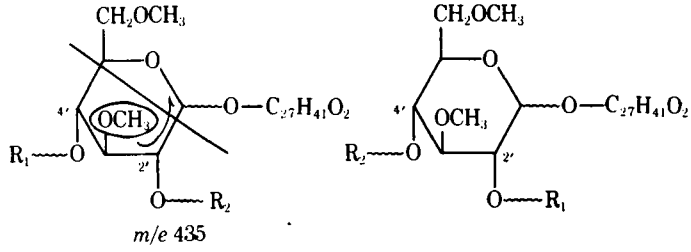
Glycoside	ED_{50}, μg/ml P-388	L-1210	KB
I	0.94	0.14	0.16
VI	0.22	0.43	0.029
Synthetic 3-[glucosyl]-25(*R*)-spirost-5-en-3β-ol	23.9	65.7	12
Synthetic 3-[maltosyl]-25(*R*)-spirost-5-en-3β-ol	30.8	58.9	29

[a] For the P-388 and L-1210 tissue cultures, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (NSC 95441) was used as the positive control compound, tested at 10, 5, 2.5, 1.25, and 0.625 μg/ml. For synthetic compounds, an ED_{50} value of ≤ 4 μg/ml is considered to be significant cytotoxic activity. For the KB tissue culture test system, mercaptopurine monohydrate was used as the standard.



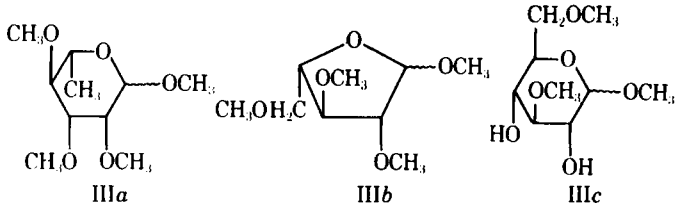
Scheme I

nosyl and tri-*O*-methylarabinosyl groups are attached to the C-2′ and C-4′ positions of the D-glucose moiety.

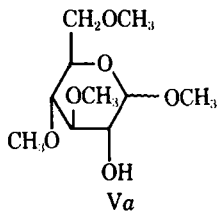


R_1 = tri-*O*-methyl-L-arabinosyl
R_2 = tri-*O*-methyl-L-rhamnosyl

The position of the sugar linkage was established by GLC analyses (7) of the methyl glycosides derived from methanolysis of III, which showed the presence of IIIa–IIIc. The identification of these methyl glycosides was made by comparison of their GLC retention times and mass spectra (10) with samples synthesized by published procedures (13, 14). These results were consistent with the supposition that rhamnose is attached to either the C-2′ or C-4′ position of glucose.



IIIa IIIb IIIc



Va

The exact locations of the sugar linkages in I were deduced by the partial acid hydrolysis of I, which afforded 3-[β-D-glucopyranosyl]-25(*R*)-spirost-5-en-3β-ol and the prosapogenin IV. Paper chromatographic analysis of the sugars in the acid hydrolysate of IV showed only D-glucose and L-rhamnose, indicating that L-arabinose had been cleaved off during the partial hydrolysis of I. Permethylation of IV afforded V. Methanolysis of V gave two methyl glycosides, which were separated by column chromatography and characterized as IIIa and, more importantly, Va. This identification was accomplished by comparison of GLC retention times and mass spectral data with synthetic samples prepared by published procedures (10).

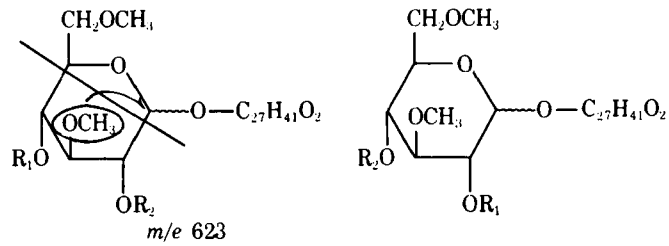
These experimental data can only be interpreted to mean that rhamnose is attached to the glucose C-2′ position. Hence, the arabinosyl unit must be attached to the glucose C-4′ in I.

With the sugar linkage sites established, the stereochemistry of the glycosidic linkages in I was then studied. The NMR spectrum of V showed signals at 4.26 (d, 1H, J = 7 Hz) and 5.15 (s, 1H) ppm. The former signal corresponds to the axial anomeric proton of the tri-*O*-methyl-D-glucopyranosyl moiety, and the latter corresponds to the equatorial anomeric proton of the tri-*O*-methyl-L-rhamnopyranosyl moiety. Thus, D-glucose is linked to II *via* a β-glycosidic bond, whereas L-rhamnose is linked to D-glucose *via* an α-glycosidic linkage.

The NMR spectrum of III exhibited signals at 4.36 (d, 1H, J = 7 Hz), 5.15 (s, 1H), and 5.18 (s, 1H) ppm. The signal at 5.15 ppm is characteristic of the equatorial anomeric proton in the tri-*O*-methyl-L-arabinosyl moiety, confirming that L-arabinose is attached to C-4′ of the D-glucose unit *via* an α-glycosidic linkage. The calculated and experimental molecular rotation values (16) for IV and I are in good agreement (Table I), further substantiating the stereochemical assignment.

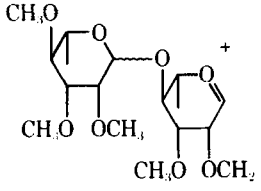
Hydrolysis of the saponin VI afforded II, D-glucose, and L-rhamnose. GLC analyses of the trimethylsilyl derivatives of these sugars revealed that D-glucose and L-rhamnose were present in a 1:3 ratio. The mass spectrum of VI showed prominent peaks at *m/e* 147, 414, 576, and 722. The ions at *m/e* 414 and 576 indicated that D-glucose was linked directly to II as 3L-rhamnose–D-glucose–II.

Methanolysis of the permethylated derivative VII gave three types of methyl sugars, which were identified as IIIa, methyl 2,3-di-*O*-methyl-L-rhamnopyranoside (VIIa), and IIIc. Thus, VII may be represented by one of the following structures.



R_1 = tri-*O*-methyl-L-rhamnopyranosyl
R_2 = tri-*O*-methyl-L-rhamnopyranosyl(1→4)-di-*O*-methyl-L-rhamnopyranosyl

The mass spectrum of VII gave prominent ions at *m/e* 189, 363, and 623, in accord with this assignment. The peak at *m/e* 189 may be rationalized in terms of the two terminal tri-*O*-methyl-L-rhamnopyranosyl units; the ion at *m/e* 363 corresponds to the tri-*O*-methyl-L-rhamnopyranosyl(1 → 4)-di-*O*-methyl-L-rhamnopyranosyl ion. The ion at *m/e* 623 strongly suggests C-2′ and C-4′ substitutions of the D-glucosyl unit.



m/e 363

Partial acid hydrolysis of VII afforded VIII, whose mass spectrum gave prominent ions at *m/e* 189, 379 and 796 (M^+), indicating that a tri-*O*-methyl-L-rhamnopyranosyl(1 → 4)-di-*O*-methyl-L-rhamnopyranosyl group was cleaved during hydrolysis. Since methylation of VIII afforded V, one may logically conclude that the L-rhamnopyranosyl(1 → 4)-L-rhamnopyranosyl group must be attached to C-4′ and that the L-rhamnopyranosyl group may be attached to C-2′ of the D-glucopyranosyl moiety of VII.

The NMR spectrum of VII showed a doublet at 4.4 ppm (1H, J = 7 Hz) and two broad singlets at 5.03 (1H) and 5.21 (2H) ppm. The doublet corresponds to the axial anomeric proton of D-glucose, and the singlets correspond to the equatorial anomeric protons of the three L-rhamnose units. Thus, the D-glucose unit is attached to II *via* a β-glycosidic linkage and to the three L-rhamnose residues *via* α-glycosidic bonds, and the chemical structure of this tetraglycoside is represented by VI.

The cytotoxic activities of I and VI are shown in Table II. Both saponins were quite effective against P-388, L-1210, and 9KB tissue culture systems. To demonstrate that this cytotoxic activity is not simply due to a detergent effect, glucoside and maltoside of II were prepared and evaluated in these test systems. Since both synthetic saponins were much less active, it appears that some unique structural features are required for optimal cytotoxic activity.

Extracts of *Dioscorea* have been widely used for the treatment of a variety of tumors in China, India, South America, and Southeast Asia (17). After completion of these studies, it was noted that I and VI had been isolated from the dried rhizomes of *Paris polyphylla* SM. obtained in a market of Katmandu, Nepal (18).

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ACKNOWLEDGMENTS

Supported in part by National Institutes of Health Grant AM 04874. The authors thank Professor David Perlman for the cytotoxic testing.

NOTES

Phosphodiesterase Inhibition by Succinic and Related Acid Biphenylalkyl Monoesters

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Received September 22, 1978, from the *Department of Pharmaceutical Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298.* Accepted for publication December 7, 1978.

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Abstract □ Biphenylalkyl monoesters of succinic and related acids represent a potent new class of cyclic AMP phosphodiesterase inhibitors. The biphenyl group is necessary for potent inhibition. The spatial relationship of the carboxyl and ester groups and alkyl chain length are important to inhibitory potency.

Keyphrases □ Succinic acid biphenylalkyl monoesters—phosphodiesterase inhibition, synthesis, structure–activity relationships □ Cyclic AMP phosphodiesterase—inhibitors, succinic acid biphenylalkyl monoesters, synthesis, structure–activity relationships □ Phosphodiesterase inhibitors—succinic acid biphenylalkyl monoesters, synthesis, structure–activity relationships

Tissue cyclic AMP levels appear to be regulated by two enzymes, adenylate cyclase and phosphodiesterase, and by cellular extrusion. Phosphodiesterase catalyzes the hydrolysis of cyclic AMP to 5′-AMP. Because phosphodiesterase inhibitors can increase tissue cyclic AMP levels, these compounds are of interest as biochemical tools and as potential therapeutic agents (1–3).

Most potent cyclic AMP phosphodiesterase inhibitors are nitrogen-containing heterocycles, *i.e.*, theophylline, 3-isobutyl-1-methylxanthine, papaverine, and 4-(3-butoxy-4-methoxyphenyl)-2-imidazolidinone[1], and most contain either a 6,5- or 6,6-fused heterocyclic ring (4–6). Only a few reports described the effects of organic acids on phosphodiesterase. Citrate at high concentrations (12 m*M*) inhibited the enzyme (7). Ethacrynic acid was reported to be one-half as potent as theophylline against the enzyme from beef heart (8). Recently, acidic anti-inflammatory agents were reported to inhibit phosphodiesterase (9–11). Enzyme activation by the lipoidal organic acid, stearic acid, was observed (12), as was inhibition by unsaturated fatty acids (13).

1-(4-Biphenylyl)pentyl hydrogen succinate is a hypo-

[1] Ro 20-1724.

0022-3549/79/0700-0903$01.00/0