penicillin derivatives when log P for the unionized form is used rather than log P of the apparently more appropriate ionized form. Similar behavior was found in relating log P to the extent of binding of carbenicillin and ticarcillin to serum proteins[2]. Possibly this effect is coincidental due to ionic binding by the side-chain carboxy group to an extent approximately sufficient to offset the low hydrophobic binding due to the ionic nature of this group.

Nishida *et al.* (13) reported binding constants (k_{rel}) relative to benzylpenicilloic acid for binding to benzylpenicilloyl specific antibodies of some penicilloic acids not studied in this work. Included were the penicilloic acids of *o*-, *m*-, and *p*-chloro- and *o*-, *m*-, and *p*-nitrobenzylpenicillins and of methyl- and ethylpenicillins. Equation 2 was used to calculate values of log (1/C) (and of k_{rel} using the experimental C value for benzylpenicilloic acid) for these compounds. This equation cannot distinguish the effect of varying the substituent position in the benzene ring, but values were calculated for chloro- and nitrobenzylpenicilloic acids using the appropriate MV values (131.1 and 143.7, respectively) and log P values (2.47 and 1.48, respectively) calculated from those of penicillin G with Hansch π values of 0.71 and −0.28 for chloro and nitro, respectively (14). The k_{rel} values obtained were 1.02 and 0.06 for the chloro and nitro compounds, respectively. These values are reasonably close to the experimental values of 1.36–5.17 for the three chloro compounds and 0.115–0.33 for the three nitro compounds, especially when it is recalled that Nishida *et al.* (13) used a fivefold dilution technique for their determinations so their results may not be very precise.

The log P value for methylpenicillin was calculated in two ways: (*a*) from log P penicillin G minus π phenyl, 1.76 − 1.89 = −0.13; and (*b*) from log P heptylpenicillin (15) minus 6 π methylene, 3.32 − 3.0 = 0.32. The mean value of 0.22 is used, and log P of ethylpenicillin then becomes 0.72 by addition of π methylene. Substitution of these values with MV values of 25.9 and 48.1 in Eq. 2 leads to k_{rel} values of 2.16 and 1.96 for methyl- and ethylpenicilloic acids, respectively.

These values are very different from Nishida *et al.*'s experimental values of 0.009 and 0.016. Thus, penicilloic acids with such small side chains are outside the range of applicability of Eq. 2. Intuitively, it seems probable that Eq. 2 will be applicable only to a limited range of penicilloic acids, presumably with log P and MV values centered about those of benzylpenicilloic acid because the antibodies were raised to a benzylpenicilloyl antigen. Experiments with antibodies raised to other penicilloyl antigens would be interesting in this context.

REFERENCES

(1) E. Kutter and C. Hansch, *Arch. Biochem. Biophys.*, **135**, 126(1969).

(2) A. E. Bird and A. C. Marshall, *J. Chromatogr.*, **63**, 313(1971).

(3) G. G. Nys and R. F. Rekker, *Chim. Ther., No. 5*, **1973**, 521.

(4) T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.*, **86**, 5175(1964).

(5) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8**, 150(1965).

(6) J. R. Partington, "An Advanced Treatise on Physical Chemistry," vol. II, Longmans, London, England, 1951, p. 22.

(7) O. Exner, *Collect. Czech. Chem. Commun.*, **32**, 1(1967).

(8) G. Kortum, W. Vogel, and K. Andraussow, "Dissociation Constants of Organic Acids in Aqueous Solution," Butterworth, London, England, 1961.

(9) C. Hansch and J. M. Clayton, *J. Pharm. Sci.*, **62**, 1(1973).

(10) C. Hansch, in "Drug Design," vol. I, E. J. Ariëns, Ed., Academic, New York, N.Y., 1971, p. 308.

(11) C. Hansch and C. Silipo, *J. Med. Chem.*, **17**, 661(1974).

(12) C. Hansch, in "Structure–Activity Relationships," vol. I, C. J. Cavallito, Ed., Pergamon, Oxford, England, 1973, p. 75.

(13) K. Nishida, K. Kinoshita, T. Atsumi, K. Shibata, and Y. Horiuchi, *Immunochemistry*, **9**, 1195(1972).

(14) C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani, and E. J. Lien, *J. Med. Chem.*, **16**, 1207(1973).

(15) A. E. Bird and J. H. C. Nayler, in "Drug Design," vol. II, E. J. Ariëns, Ed., Academic, New York, N.Y., 1971, p. 277.

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Cytotoxic Principles of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)

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Abstract □ Systematic fractionation of the cytotoxic extracts of the roots of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae) on silica gel column chromatography led to the isolation of three cardenolides: cymarin, strophanthidin, and a strophanthidin glycoside (XS-89). Also isolated during the investigation were β-sitosterol-β-D-glucoside, a mixture of α- and β-amyrins, a mixture of alkanols, and a mixture of plant sterols.

Keyphrases □ *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)—isolation and identification of cytotoxic principles □ Cytotoxicity—isolation and identification of cymarin, strophanthidin, and strophanthidin glycoside from *Parquetina nigrescens* □ Cardenolides—isolation and identification from *Parquetina nigrescens*

Parquetina nigrescens (Asclepiadaceae), a woody shrub native to Africa, is sometimes referred to as *Periploca nigrescens* (1). Previous studies (2–5) showed it to contain cardiac glycosides of the strophanthidin type. Strophanthidiol-β-glucoside acetates, strophanthidin, strophanthidiol, 16-hydrostrophanthidin, convallatoxin, 17α-strophanthidin, 16-acetoxystrophanthidin, 16-acetoxystrophanthidin rhamnoside, 16-dehydrostrophanthidin, 16-dehydrostrophanthidol, nigrescigenin, tetra-*O*-acetylstrophanthidin-β-D-glucoside, and several incompletely identified cardenolides have been isolated from this plant.

Other species of the genus have also been investi-

gated for the presence of cardenolides (6–8). Periplogenin and cymarose have been isolated from *P. calophylla* (6) and *P. sepium*. Periplogenin and periplocymarin (7) as well as periplocin (8) were found in *P. graeca*.

P. graeca has folkloric uses as a herbal or arrow poison (9), and *P. sepium* has been an ingredient of a cure-all drug in Chinese herbal medicine (2, 10). Pharmacological studies on various species of the genus have centered on their cardiotonic effects (11–13).

DISCUSSION

A 50% alcoholic extract of the roots of *P. nigrescens* was found to be cytotoxic[1] ($ED_{50} < 8.8 \times 10^{-1}$ μg/ml). However, this extract and fractions from subsequent workups of the plant material were inactive when tested in the P-388 leukemia system in mice.

TLC of the alcoholic extract indicated the presence of several cardenolides (positive reactions to the Kedde spray reagent).

Partition of a 95% alcoholic extract between water and chloroform resulted in the isolation and identification of β-sitosterol-β-D-glucoside and cymarin from the chloroform-soluble fraction (C). The isolation of cymarin represents the first reported occurrence of this cardenolide in *P. nigrescens*.

Chromatographic separation of a 50% alcoholic extract of the marc resulted in the isolation of strophanthidin and a novel cardenolide, which was partially characterized as a strophanthidin glycoside.

The petroleum ether solubles (Fraction A) yielded mixtures of alkanols, phytosterols (campesterol, stigmasterol, and β-sitosterol), and triterpenes (α- and β-amyrins).

Biological evaluation of the isolates demonstrated that the cytotoxic activity of this plant was due, at least in part, to the three cardenolides present.

EXPERIMENTAL[2]

Plant Material—The plant material[3] was collected in Ghana during March 1971. The roots were collected, air dried, and ground to a coarse powder.

Extraction and Initial Fractionation—A 4.5-kg sample of the plant material was defatted with petroleum ether (bp 30–60°), and the extractive was concentrated *in vacuo* to yield 119 g of Fraction A. The defatted marc was air dried and then continuously extracted with 95% ethanol to give Fraction B (199 g).

A 100-g portion of Fraction B was taken to a syrupy consistency and partitioned between chloroform and water to give Fractions C (24 g) and D (70 g), respectively.

The 95% ethanol-exhausted marc was air dried and continuously extracted with 50% ethanol to yield Fraction E (64 g).

Separation of Fraction C—Fraction C was subjected to column chromatography on 600 g of silica gel PF-254[4], using benzene–95% ethanol (3:1) as the eluent. Then 120 15-ml fractions were collected, and fractions were combined on the basis of TLC monitoring.

Isolation and Identification of β-Sitosterol Glucoside—Fractions 33–44 were combined, filtered, and taken to dryness. The residue was dissolved in hot dioxane and, upon cooling, yielded a copious white amorphous precipitate. The substance had a melting point of 260–270°, gave a transparent UV spectrum, and showed IR absorptions at 3425 (s, OH), 2980 (s, CH_3 stretch), 2890 (w), 1470 (w), 1380 (w), 1180 (s), and 1025 (s) cm^{-1}.

The mass spectrum was superimposable with that of β-sitosterol, but the sample did not vaporize until 270° *in vacuo*. The latter point, plus the compound's different TLC, IR, and solubility properties as compared to reference β-sitosterol, suggested that the isolate was a glycoside. A comparison of the IR spectrum with that of reference β-sitosterol-β-D-glucoside[5] showed them to be superimposable. Furthermore, a mixed melting-point determination did not lower the melting-point range of the mixture. Therefore, the isolate was identified as β-sitosterol-β-D-glucoside.

Isolation and Identification of Cymarin—Fractions 47–50 were combined and dried *in vacuo* to a residue (500 mg), which was rechromatographed on a column of 75 g silica gel PF-254 and eluted with benzene–absolute ethanol (3:1). Forty-one 10-ml fractions were collected, and fractions 33–37 were combined on the basis of their TLC patterns. The dried, pooled fraction (200 mg) was dissolved in methanol, and ether was added until a cloudy appearance was observed.

The flask was stoppered and clear crystalline needles (72 mg) were obtained after 4 hr. These needles gave a Kedde positive reaction and had a melting point of 138–141°. Mass spectra of the isolate were run at 15 and 70 ev and showed fragmentation patterns consistent with those of cymarin (14). In addition, the NMR, UV, and IR spectra were superimposable with those of reference cymarin[6], and a mixed melting-point determination showed no depression.

Separation of Fraction E—Fraction E, the 50% alcoholic extract of the 95% ethanol-exhausted marc, was partitioned between chloroform and water. The chloroform layer was shown to be devoid of Kedde-positive material. The aqueous layer was evaporated *in vacuo*, and the residue (250 mg) was chromatographed on a 75-g silica gel PF-254 column, eluting with benzene–methanol (8:1). Forty 10-ml fractions were collected.

Isolation of Strophanthidin—Column fractions 15–20 were combined on the basis of their TLC patterns and taken to dryness. The residue (70 mg) was dissolved in hot methanol, ether was added to slight turbidity, and the flask was stoppered and allowed to stand overnight. The resulting colorless crystals (50 mg) were recrystallized from methanol–ether to afford an analytical sample which gave both positive Kedde and Liebermann–Burchard reactions.

The product had a melting point of 140–142° and exhibited NMR, UV, and IR spectra superimposable with those obtained for reference strophanthidin[7]. The mass spectrum of the isolate was consistent with that of strophanthidin (14). That this isolate was strophanthidin was further supported by an undepressed mixed melting-point determination.

Isolation and Characterization of the Strophanthidin Glycoside (XS-89)—Column fractions 21–25 were combined and evaporated, and the residue (80 mg) was dissolved in methanol. Water was added to the methanolic solution until it became cloudy, and the flask was stoppered and set aside in the refrigerator. The crystalline needles that formed were collected and dried *in vacuo* to give a total weight of 62 mg. The mother liquor was concentrated by slow evaporation at room temperature, and a second crop was harvested (8 mg). This compound was subsequently determined to be a new cardenolide possessing cytotoxic activity.

The isolate had a melting point of 163–168° and exhibited a UV spectrum at λ_{max} 218 nm ($\log \epsilon^{1\%}_{1cm}$ 2.47). IR spectral absorptions were observed at 3500 (s, OH), 2980 (s, CH_3 stretch), 2910 (w), 1750 (s, α,β-unsaturated lactone), 1725 (w, aldehyde), 1500 (m),

[1] The extracts were tested through the auspices of the Drug Research and Development branch of the National Cancer Institute. An extract is considered cytotoxic if it exhibits an $ED_{50} \leq 20$ μg/ml, and an isolate is considered active it it shows an $ED_{50} \leq 4$ μg/ml in the 9KB cell culture *in vitro* (17).

[2] All melting points reported were determined using a Kofler hot-stage melting-point apparatus and are uncorrected. UV spectra were recorded using a Beckman model DB-G spectrophotometer with methanol as the solvent. IR spectra in KBr were recorded using a Beckman model 18-A spectrophotometer with polystyrene calibration at 1601 cm^{-1}. Absorption bands are recorded in wave numbers ($centimeters^{-1}$) and intensities as s (strong), m (medium), and w (weak). NMR spectra were recorded in deuterochloroform on Varian Associates models A-60 and T-60 instruments using tetramethylsilane as the internal standard. Chemical shifts are reported in parts per million (δ, ppm). Mass spectra were determined using a Hitachi Perkin-Elmer model RMU-60 spectrometer; samples were introduced as solids. GLC analysis was carried out using a Perkin-Elmer model 881 gas chromatograph with helium as the carrier gas. The glass column was coiled, 1.8 m, 0.6 cm (6 ft, 0.125 in.) o.d., packed with a 5% OV-101 stationary phase on Gas Chrom Q, 100–120 mesh.

[3] The plant material was collected and identified by Mr. O. B. Dokosi, University of Ghana. Herbarium specimens are on file in the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

[4] E. Merck, Darmstadt, Germany.

[5] Kindly supplied by Dr. C. R. Mitra, Assistant Director, National Botanic Gardens, Lucknow, India.

[6] Kindly supplied by Professor R. Hänsel, Director Freie Universität Berlin, Institut für Pharmakognosie, Berlin, Germany.

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and 1080 (w) cm^{-1}. The NMR spectrum in deuterochloroform showed major signals as singlets at δ 0.80 (3H, angular methyl), 4.83 (2H, methylene protons of lactone ring), 5.90 (1H, olefinic proton of lactone ring), and 10.00 (1H, aldehyde).

The mass spectrum showed the expected fragmentation pattern for a strophanthidin-type cardenolide (14), with significant fragments being recorded at *m/e* 368 (8.3), 358 (4.1), 340 (45.8), 322 (31.6), 308 (4.1), 215 (4.1), 197 (7.5), 188 (20.8), 160 (15.0), 147 (100.0), 145 (12.0), and 131 (40.0).

A sample of the isolate (2 mg) was hydrolyzed by refluxing overnight in 20 ml of a mixture of 95% ethanol–water–concentrated hydrochloric acid (85:15:10). The solution was taken to dryness *in vacuo* and extracted with a small volume of water. The aqueous solution was filtered and mixed with an ion-exchange resin[8] (OH-form) until it was neutral to pH paper. The neutralized solution was filtered, dried *in vacuo*, and trimethylsilylated[9].

GLC of the silylated glycone portion demonstrated the presence of glucose. Several other peaks were also present, but their identity could not be discerned. However, fructose, digitoxose, galactose, mannose, arabinose, and rhamnose were absent.

TLC of the water-insoluble residue remaining from the hydrolysis demonstrated the presence of a component which was Kedde test positive and identical with reference strophanthidin in two solvent systems [1-butanol–acetic acid–water (4:4:1) and chloroform–methanol (8:1)]. These results, coupled with the observed mass spectral fragmentation pattern of the parent glycoside, indicated the aglycone to be strophanthidin. Attempts are underway to elucidate the structure of this compound.

Separation of Fraction A—Ten grams of the petroleum ether-soluble extract was dissolved in boiling acetone. Upon cooling, a copious, white, plate-like precipitate formed, which was collected and set aside. It exhibited a low broad melting-point range (64–72°). GLC analysis indicated the presence of several compounds which were not identified.

The mother liquor was evaporated, and the residue (6.24 g) was chromatographed on silica gel PF-254 (300 g), using benzene–ethyl acetate–acetone (10:1:1) as the eluent. A total of 38 fractions of 15 ml each was collected.

Isolation of Alkanol Mixture—Column fractions 20–24 were combined on the basis of TLC and evaporated. The residue (2.5 g) was dissolved in benzene, and acetone was added. The resulting white, plate-like precipitate, mp 60–68°, exhibited a transparent UV spectrum and the IR spectrum was typical of an alkanol (15). On comparison with reference alkanols, GLC analysis indicated the presence of 1-docosanol, 1-tetracosanol, and 1-hexacosanol.

Isolation of Triterpene Mixture—Column fractions 28–32 were combined and evaporated. This residue (1.9 g) was dissolved in chloroform, and a mixture of methanol–water (9:1) was added until a cloudy solution persisted. The microcrystalline precipitate that formed was collected and dried. The isolate (1.2 g), mp 125–130°, had a transparent UV spectrum, and it gave a positive Liebermann–Burchard test for triterpenes.

The mass spectrum showed a molecular ion at *m/e* 426 and a base peak, indicative of the amyrin series of triterpenes (16), at *m/e* 218. GLC analysis of this sample with reference standards showed the isolate to be a mixture of 70% α-amyrin and 30% β-amyrin.

Isolation of Phytosterols—Column fractions 36–38 were combined and dried *in vacuo*. On dissolving the residue (0.23 g) in 95% ethanol, small needle-like crystals (50 mg), mp 132–137°, were obtained. The isolate gave a positive Liebermann–Burchard test and exhibited a transparent UV spectrum. The IR spectrum (KBr) was identical to that of reference β-sitosterol[10]. The mass spectrum showed a molecular ion at *m/e* 414 and a base peak at *m/e* 218.

GLC of the sample at 260°, however, indicated the isolate to be composed of three constituents. These were tentatively identified as campesterol (20%), stigmasterol (7%), and β-sitosterol (73%) by comparison with chromatographically pure reference sterols, using cholestane as the internal standard.

[8] Amberlite IRA-410.
[9] SIL-PREP, Applied Science Laboratories, State College, Pa.
[10] ICN Nutritional Biochemicals Corp., Cleveland, OH 44128

Table I—Cytotoxic Results of Isolates from *P. nigrescens*

Isolate	9KB Cell Culture Test[a], μg/ml
β-Sitosterol-β-D-glucoside	$>1 \times 10^{1}$
Cymarin	$<1 \times 10^{-1}$
Strophanthidin	$<1 \times 10^{0}$
Unidentified strophanthidin glycoside (XS-89)	$<2.5 \times 10^{-1}$
Mixture of alkanols	$>1 \times 10^{2}$
Mixture of α- and β-amyrins	Not tested
Phytosterol mixture	$>4 \times 10^{1}$

[a] A compound is deemed active if it exhibits an $ED_{50} \leqq 4$ μg/ml.

Biological Activity—The results of biological evaluation indicated that the three cardenolides isolated, cymarin, strophanthidin, and the strophanthidin glycoside (XS-89), are cytotoxic but inactive in the P-388 lymphocytic leukemia (3PS) system (Table I). All other isolates tested were inactive in both systems.

REFERENCES

(1) "Index Kewnsis," Suppl. XIV, Clarendon Press, Oxford, England, 1961–1965, p. 99.

(2) R. Mauli and C. Tamm, *Helv. Chim. Acta*, **40**, 299(1957).

(3) T. Reichstein, *ibid.*, **48**, 1659(1965).

(4) R. Berthold, W. Wehrli, and T. Reichstein, *ibid.*, **48**, 1634(1965).

(5) E. Schenker, A. Hunger, and T. Reichstein, *ibid.*, **37**, 1004(1954).

(6) S. Sakuma, H. Okino, and J. Shoji, *Yakugaku Zasshi*, **89**, 979(1969).

(7) G. Netien, F. Kozjek, and M. C. Favre, *C. R. Soc. Biol. Fil.*, **161**, 1718(1967).

(8) N. Komissarenko and R. B. Bagirov, *Izv. Akad. Nauk Azerb. SSR, Ser. Biol. Nauk*, **5**, 122(1969); through *Chem. Abstr.*, **73**, 123456t.

(9) J. Watt and M. Breyer-Brandwijk, "The Medicinal and Poisonous Plants of Southern and Eastern Africa," 2nd ed., E. and S. Livingston Ltd., London, England, 1962, p. 70.

(10) S. Sakuma, S. Kawanishi, and J. Shoji, *Chem. Pharm. Bull.*, **16**, 326(1968).

(11) "United States Dispensatory XXIV," A. Osol and G. E. Farrar, Eds., J. B. Lippincott, Philadelphia, Pa., 1947, p. 1546.

(12) K. D. Sargin, *Byull. Nauch.-Issled. Khim.-Farm. Inst.*, **1930**, 9(1930); through *Chem. Abstr.*, **26**, 6014[2].

(13) M. Jacque, *Bull. Soc. Pharm. Nan.*, **82**, 9(1969).

(14) P. Brown, F. Brüschweiler, G. R. Pettit, and T. Reichstein, *Org. Mass Spectrom.*, **5**, 573(1971).

(15) R. T. Conley, "Infrared Spectroscopy," 2nd ed., Allyn and Bacon, Boston, Mass., 1972, p. 130.

(16) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," vol. 2, Holden-Day, San Francisco, Calif.,1964, p. 222.

(17) R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep. Part 3*, **3**, 1(1972).

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