90–100° for 4.5 hours, 40 ml. of absolute ethanol added, and the mixture cooled overnight at 10°. The product was collected and recrystallized repeatedly from 80% ethanol; the yield was 0.8 g. (30%).

In preliminary experiments, it was shown that choline sulfate supported good growth of *P. chrysogenum* when added to the culture medium as the sole source of sulfur. Choline sulfate was then compared with sodium sulfate as a source of sulfur for penicillin biosynthesis. Experiments were performed in which equimolar amounts of each compound were added to the medium, but in which one compound only was labeled with S^{35}. Determinations of the specific activity of the penicillin formed provided a measure of the extent to which each of the two compounds had been utilized. It can be estimated from the data of Table I that 80–90% or the penicillin sulfur is derived from inorganic sulfate.

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Saponin from Ladino Clover (*Trifolium repens*)[1,2]

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Saponins isolated[4] from water extracts of alfalfa have been shown to cause typical symptoms of bloat in ruminants.[5] These results suggested that other bloat-producing forages be examined for similar compounds.

Experiments with water extracts of ladino clover (*Trifolium repens*), designed to isolate the saponin as the cholesterol addition product as had been done with alfalfa, showed that the reaction of cholesterol with the water extract yielded only small amounts of cholesteride. However, extraction of either green or dry material with ethanol–water solutions yielded a mixture from which a saponin could be crystallized readily; this proved to be a mixed calcium–magnesium salt of at least three saponins. Hydrolysis of the mixture yielded glucose, galactose, xylose and rhamnose plus a mixture of neutral sapogenins. Chromatographic separation of the sapogenins furnished soyasapogenol B and soyasapogenol C; traces of a third substance, possibly soyasapogenol A, also were encountered. It would appear, therefore, that these sapogenins may be more common among plants than had hitherto been suspected.

Experimental[6]

Isolation of Saponin.—Freshly cut ladino clover (150 kg.) was immersed in 360 liters of 95% alcohol for 2 days, the mixture was filtered and the filtrate was concentrated to about 30 liters. The cooled solution along with some dark green insoluble lipid fraction was transferred to large separatory funnels. The residue was transferred with diethyl ether, followed by a small quantity of water. The funnels were inverted gently several times (vigorous shaking gave emulsions) until most of the chlorophyll and lipid material were in solution. After about 30 minutes the aqueous layer was drained. The saponin crystallized spontaneously in shimmering micro-plates. The ether layer then was washed repeatedly with small quantities of water and the water solutions were combined and allowed to stand for a length of time to obtain additional crops of crystalline saponin. The aqueous phase was centrifuged and the crystalline saponin was washed in centrifuge tubes several times with small quantities of water. The saponin finally was washed with acetone until no more colored material was removed. This yielded white, crystalline saponin (70 g. after drying *in vacuo* at 60°), representing 0.23% of the dry weight of ladino clover.[7]

This material after recrystallization from aqueous methanol and drying *in vacuo* at 100°, decomposed at *ca.* 225°, $[\alpha]^{25}D$ −3.3° (CH_3OH).

Anal. Found: C, 59.5; H, 8.37; N, 0.02; sulfated ash, 6.70. The ash contained calcium and magnesium equivalent to 1.65 and 0.15%, respectively, of the saponin.

Dowex-50 Treated Saponin.—The mixed calcium–magnesium salts of saponin (2 g.) were dissolved in 1800 ml. of 50% ethanol and poured through a column containing 50 g. of Dowex-50 resin. The filtrate was concentrated to 100 ml., and the free acids of the saponin were filtered, washed with water, and dried at 100° in a vacuum; yield 1.93 g.

Anal. Found: C, 61.2; H, 8.39; sulfated ash, 0.06; av. equiv. wt., 963.

When the saponin was hydrolyzed, neutral sapogenins, several sugars and an unknown acid were liberated. The sugars were identified by paper chromatography as glucose, galactose, xylose and rhamnose. The acid has not been identified, but paper chromatograms indicate that it is not galacturonic, glucuronic or gluconic acid.

Properties of Ladino Clover Saponin.—The saponin preparation was poorly soluble in water (0.33 g./liter), moderately soluble in 50% alcohol, and slightly soluble in higher concentrations of alcohol. It did not hemolyze red blood cells at a concentration of 1% and did not kill fish (*Lebistes reticulatus*) in a saturated aqueous solution. In the dry crystalline state it gave a red color with sulfuric acid. When tested with isolated strips of rabbit intestine, ladino saponin in a concentration of 10 mg./50 ml. of saline bath caused an abrupt increase in tonus, a rapid loss in peristalsis, and early damage to the tissue.

Isolation of Sapogenins.—A mixture of 10 g. of saponin, 600 ml. of 50% ethanol and 20 ml. of concentrated sulfuric acid was refluxed for 68 hours, cooled, filtered and the solid was washed free of acid and dried. This material then was dissolved in 800 ml. of ether and decolorized with charcoal. Two recrystallizations from methanol–chloroform yielded 1.28 g. of sapogenins (m.p. *ca.* 235°) and an additional 0.7 g. could be secured from the original filtrate by concentration and ether extraction. The sapogenin mixture was soluble in the common organic solvents (*e.g.*, chloroform, ether, alcohol) and gave a red color with sulfuric acid and the Liebermann–Burchard reagent and a pale yellow color with tetranitromethane. Attempts to titrate the sapogenins with alkali indicated that no acidic groups were present.

The sapogenins (20 g.) dissolved in 1 liter of benzene were chromatographed on a column (36 mm. × 48 cm.) of deactivated alumina[8] and eluted with increasing concentrations of methanol in benzene. Three definite fractions (m.p. 239–241°, 258–260° and 318–320°) were eluted with, respectively, 0.5, 2.5 and 100% of methanol. These melting points are in reasonable agreement (*cf.* Table I) with those reported[9,10] for soyasapogenols C, B and A isolated from soya beans. Soyasapogenol B (*ca.* 75%) and soyasapogenol C (*ca.* 20%) were identified unambiguously as shown below, while no additional work was done with the presumed soyasapogenol A which was present only in trace quantities.

(1) This note is to be considered Paper XVIII in the series "Terpenoids" from Wayne University.

(2) Article not copyrighted.

(3) Wayne University, Detroit, Michigan.

(4) E. D. Walter, G. R. Van Atta, C. R. Thompson and W. D. Maclay, THIS JOURNAL, **76**, 2271 (1954).

(5) I. L. Lindahl, A. C. Cook, R. E. Davis and W. D. Maclay, *Science*, **119**, 157 (1954).

(6) Melting points were determined on the Kofler block. Unless noted otherwise, rotations were measured in chloroform solution.

(7) Crystalline saponin also was isolated in small quantity from alfalfa by the same procedure. This saponin appeared to be identical with that from ladino clover as indicated by X-ray diffraction studies, infrared absorption, and by microscopic examination of the crystals. Potter and Kummerow (*Science*, **120**, 224 (1954)) isolated a saponin preparation from alfalfa which they found yielded at least three triterpenoid sapogenins, one of which was identical with soyasapogenol B.

(8) Alorco alumina grade F-20 was deactivated by shaking 300 g. of alumina with hexane containing 12 ml. of 10% aqueous acetic acid for 3 hours.

(9) E. Ochiai, K. Tsuda and S. Kitagawa, *Ber.*, **70B**, 2083 (1937).

(10) A. Meyer, O. Jeger and L. Ruzicka, *Helv. Chim. Acta*, **33**, 672 (1950).

TABLE I

COMPARISON OF LADINO SAPOGENINS WITH THE CORRESPONDING SOYASAPOGENOLS

		Ochiai, *et al.*	Meyer, *et al.*	Ladino sapogenins
Soy. A	M.p., °C.	311	321	318–320
	$[\alpha]_D$	+102	+103	...
Soy. B	M.p., °C.	260	259–260	260
	$[\alpha]_D$	+92	+90	+91
Soy. C	M.p., °C.	238–239	239–240	240–241
	$[\alpha]_D$	+70.7	+65	+63
B-triAC	M.p., °C.	175–176	179–180	180–181
	$[\alpha]_D$	...	+78	+78
C-diAC	M.p., °C.	198	199–200	205–207
	$[\alpha]_D$	...	+59	+58

Soyasapogenol B.—The eluates melting at 258–260° were combined and recrystallized from methanol–chloroform yielding material of m.p. 260°, $[\alpha]^{25}D$ +91°.

Anal. Calcd. for $C_{30}H_{50}O_3$: C, 78.55; H, 10.99. Found: C, 78.20; H, 10.91.

A sample was acetylated by refluxing with acetic anhydride for 2 hours. Recrystallization from chloroform–methanol yielded the **triacetate** as laths, m.p. 180–181°, undepressed upon admixture with an authentic specimen,[10] $[\alpha]^{24}D$ +78°; identity was confirmed by comparison of the infrared spectra.

Anal. Calcd. for $C_{36}H_{56}O_6$: C, 73.93; H, 9.65. Found: C, 73.80; H, 9.61.

Soyasapogenol C.—The eluates melting at 239–241° were combined and recrystallized from methanol–chloroform whereupon they exhibited m.p. 240–241°, $[\alpha]^{25}D$ +63°. The melting point was not depressed on admixture with an authentic sample.[10]

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 82.05; H, 11.02.

Acetylation with acetic anhydride–pyridine (room temperature, 20 hours) followed by crystallization from methanol–chloroform furnished the **diacetate**, m.p. 205–207°, $[\alpha]^{27}D$ +58°, which also was obtained by direct chromatography (elution with 7:3 hexane–benzene) of the acetylated sapogenin mixture.

Anal. Calcd. for $C_{34}H_{52}O_4$: C, 77.82; H, 9.99. Found: C, 78.01; H, 9.92.

Oxidation of the diacetate (160 mg.) with an equal amount of selenium dioxide in acetic acid solution in the standard manner[11] and recrystallization from methanol yielded the **Δ11,13(18),(?)15-triene** as blades, m.p. 208–211°, λ_{max}^{EtOH} 241, 249 and 259 mμ, log ε 4.20, 4.23 and 4.17; lit.,[8] m.p. 208–209°.

Anal. Calcd. for $C_{34}H_{50}O_4$: C, 78.12; H, 9.64. Found: C, 78.13; H, 9.78.

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(11) *Cf.* L. Ruzicka, G. Müller and H. Schellenberg, *Helv. Chim. Acta*, **22**, 767 (1939).

Aliphatic Esters of 3-Indoleacetic Acid. Preparation and Activity in Parthenocarpic Fruit Induction[1,2]

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The isolation and discovery of the unique plant growth regulating properties of heteroauxin soon led to various methods of biological assay. Such tests made it possible to compare other compounds, possessing biological properties similar to 3-indoleacetic acid. Kögl and Kostermans,[3] using the *Avena* curvature test, reported that 3-indoleacetic acid was more active than the methyl, ethyl, propyl or isopropyl esters. The biological action of the esters decreased as the size of the ester radical increased. This activity order has been attributed to a parallel decrease in the hydrolyzability of the ester and assumes that the esters exhibit activity only indirectly by giving rise to 3-indoleacetic acid upon hydrolysis *in vivo*.[3,4] Although logical, it has not been substantiated by experimental evidence. An equally plausible explanation[5] is the impeded transport of the ester *per se*.

Zimmerman, *et al.*,[6] employing tomato stem and petiole elongation as a criterion for biological activity, reported that methyl 3-indoleacetate as well as the methyl esters of other indole acids were more active than the corresponding free acids. Similar results have been obtained in the stimulation of parthenocarpy in the tomato,[7,8] the lower molecular weight aliphatic esters all being more active than 3-indoleacetic acid. These results have led to a study of other esters of 3-indoleacetic acid. The synthesis and properties of these new esters are described in this report.

Experimental

The esters of 3-indoleacetic acid were prepared from the acid and the appropriate alcohol, with hydrogen chloride catalyst, similar to the procedure described by Jackson[9] for the methyl and ethyl esters. The esterifications usually were carried out at room temperature except in those cases where elevated temperatures were necessary to effect solution of the reaction mixture. After esterification, the excess alcohol was removed by vacuum distillation and the residue ultimately vacuum distilled. The esters were purified further by repeated recrystallization from petroleum ether (b.p. 62–67°). Esterification with *n*-dodecyl, *n*-tetradecyl, *n*-hexadecyl and *n*-octadecyl alcohol required warming to affect solution and subsequent reaction. These esters were not vacuum distilled but were recrystallized from petroleum ether. All esters were recrystallized to a constant melting point and subjected to elemental analysis. The chemical and physical properties are given in Table I.

(1) Journal Article No. 1721 from the Michigan Agricultural Experiment Station, East Lansing.

(2) This research was supported by the Horace H. Rackham Research Endowment.

(3) F. Kögl and D. G. Kostermans, *Z. physiol. Chem.*, **235**, 201 (1935).

(4) F. W. Went and K. V. Thimann, "Phytohormones," The Macmillan Co., New York, N. Y., 1937, p. 294.

(5) S. A. Gordon, *Ann. Rev. Plant Physiol.*, **5**, 357 (1954).

(6) P. W. Zimmerman, A. E. Hitchcock and F. Wilcoxon, *Contrib. Boyce Thompson Inst.*, **8**, 105 (1936).

(7) C. T. Redemann, S. H. Wittwer and H. M. Sell, *Archiv. Biochem. Biophys.*, **32**, 80 (1951).

(8) H. M. Sell, S. H. Wittwer, T. L. Rebstock and C. T. Redemann, *Plant Physiol.*, **28**, 481 (1953).

(9) R. W. Jackson, *J. Biol. Chem.*, **88**, 659 (1930).