of water was added dropwise 3.96 ml (0.033 mole) of 30% hydrogen peroxide in 5 ml of water, the temperature being maintained below 25°. After standing for 1 hr the resultant solution was evaporated at 50° at reduced pressure and the semisolid residue that was obtained was triturated with isopropyl alcohol affording 3.32 g (80.8% yield) of off-white crystals, mp 125–128° dec, of 2-aminoethaneselenenic acid. This compound is difficult to recrystallize and decomposes after several months of storage.

Anal. Calcd for C_2H_7NOSe (140.04): C, 17.15; H, 5.03; N, 10.00; Se, 56.38. Found: C, 17.45; H, 4.52; N, 9.44; Se, 56.77; mol wt, 131.[15]

Reduction of II Hydrochloride with Hydrazine.—To a solution of 1.76 g (0.01 mole) of II hydrochloride in 40 ml of methanol was added dropwise 0.22 g of 95% hydrazine. The solution was left overnight at room temperature and then evaporated to dryness on a steam bath with the aid of a stream of nitrogen. The residue was triturated with a small quantity of cold ethanol and filtered to give 1.27 g (79.6% yield) of I dihydrochloride, mp 188–189° (lit. 188°,[16] 177–179° dec,[17] 186–188°[9]).

Nmr Spectral Determinations.—The pH dependence of the nmr spectrum of II was studied in the following manner. About 0.1-ml portions of NaOD in D_2O were added to a solution of II hydrochloride in D_2O. The nmr spectrum was scanned after each addition of base from the natural pH of II hydrochloride to *ca.* pH 11. After the last scan the solution was returned to pH 3 with hydrogen chloride in D_2O. The nmr spectrum obtained was identical with that obtained from the starting II hydrochloride.

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Amino Acids and Peptides. V.[1] Synthesis of the C-Terminal Tripeptide Sequence (A_{27}–A_{29}) of Glucagon

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Several paths have been described for the preparation of the C-terminal tetrapeptide and related portions of the hyperglycemic hormone glucagon.[2] The earliest scheme disclosed the synthesis of N-phthaloyl-L-leucyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester by stepwise use of a series of *p*-nitrophenyl esters.[3] Alternatively, a potential condensation involving N-trityl-L-leucyl-L-methionine and L-asparaginyl-L-threonine methyl ester was abandoned owing to difficulties in the hydrolysis of the corresponding N-trityl methyl ester derivative. A second major route utilized an azide sequence between N-phthaloyl-L-leucyl-L-methionyl hydrazide and L-asparaginyl-L-threonine *t*-butyl ester as well as a mixed anhydride reaction with phthaloyl-L-leucyl-L-methionine and L-asparaginyl-L-threonine *t*-butyl ester to give the same tetrapeptide fragment.[4] In a third but incomplete work, N-ethylbenzisoxazolium fluoroborate was employed as the activation agent at various stages.[5] Finally, the tripeptide moiety has been obtained in the form of both N-1-methyl-2-benzoyl-vinyl-L-methionyl-L-asparaginyl-L-O-*t*-butyl-L-threonine *t*-butyl ester and the equivalent N-trifluoroacetyl-substituted derivative.[6] The former compound was prepared from a mixed anhydride reaction between N-1-methyl-2-benzoyl-vinyl-L-methionine and L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester, while the latter product was constructed through an azide coupling with N-trifluoroacetyl-L-methionine hydrazide and the same dipeptide.

A new variant for the protected tripeptide sequence of glucagon is reported here. N-benzyloxycarbonyl-O-*t*-butyl-L-threonine *t*-butyl ester (I) was reduced to O-*t*-butyl-L-threonine *t*-butyl ester (II), which was condensed with N-benzyloxycarbonyl-L-asparagine (III) with the aid of 2-ethyl-5-phenyloxazolium-3′-sulfonate (reagent K)[7] to give N-benzyloxycarbonyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (IV). This step avoids the need for N-benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester and gives a better overall yield of compound IV. Hydrogenolysis of the dipeptide IV produced L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (V), which on reaction with the 2,4,5-trichlorophenyl ester[8] of N-benzyloxycarbonyl-L-methionine (VI) afforded crystalline N-benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (VII). The tripeptide VII was formed in lower yield by combining the dipeptide amine V with N-benzyloxycarbonyl-L-methionine in the presence of reagent K. Removal of the protecting benzyloxycarbonyl group of compound VII with excess 30% palladium-on-carbon catalyst slowly yielded the corresponding oily amine (VIII).

The electrophoretic pattern of glucagon on starch shows two characteristic compounds present in the approximate ratio 9:1. If the slower moving, major component is separated and again subjected to electrophoresis, the identical fractions reappear in the same proportion. The contaminant has about half the biological activity and *qualitatively* contains the same amino acids, suggesting that the faster moving material is derived from glucagon.[9] These observations could be attributed to a conversion of the methionine residue in glucagon by air oxidation to the equivalent sulfoxide analog. The reduced physiological activity of this latter product may be associated with steric incompatability at a specific receptor site. In order to test this assumption at a future date, it would be desirable to incorporate the sulfoxide residue into synthetic glucagon. Accordingly, the tripeptide VII was oxidized with dilute hydrogen peroxide to produce the corresponding N-benzyloxycarbonyl-L-methionyl sulfoxide L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (IX). The sulfoxide IX on thioglycolic acid treatment was easily reconverted into the parent compound VII with no loss in optical activity. This last sequence was patterned on a procedure elaborated for methionine ↔ methionine sulfoxide interconversions.[10]

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Experimental Section[11]

N-Benzyloxycarbonyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (I).—A mixture of N-benzyloxycarbonyl-L-threonine (10.9 g, 0.043 mole) and dichloromethane (100 ml) in a pressure bottle (500 ml) was cooled in an acetone–carbon dioxide bath. Concentrated sulfuric acid (1.5 ml) was gradually added to the swirled suspension, followed in turn by distilled isobutylene (100 ml). After wiring a cap onto the container, the mixture was shaken mechanically at room temperature for 66 hr. The flask was chilled and opened, and the contents were diluted with more solvent (100 ml), and then poured into excess sodium bicarbonate solution (1 *N*, 200 ml). The organic phase was removed, washed with water until neutral, dried ($MgSO_4$), filtered, and evaporated to yield N-benzyloxycarbonyl-O-*t*-butyl-L-threonine *t*-butyl ester as a yellow oil (15.9 g, 100%) [lit.[6,12,13] colorless-to-yellow oil (one to two impurities generally present)]: R_f 0.75; ν_{max}^{KBr} 2970 (CH), 1725 broad (C=O), 1390 and 1365 (*t*-butyl), and 695 (Ph) cm^{-1}; $\lambda_{max}^{ethanol}$ 252, 258, 262, 264, 267 mμ (ε 317, 370, 309, 311, 244).

O-*t*-Butyl-L-threonine *t*-Butyl Ester (II).—N-Benzyloxycarbonyl-O-*t*-butyl-L-threonine *t*-butyl ester (15.88 g, 0.044 mole) was dissolved in methanol (200 ml) containing 10% palladium-on-carbon catalyst (1.75 g) and was hydrogenated for 6.5 hr. Fresh catalyst (0.36 g) suspended in ethyl acetate (20 ml) was added and hydrogenation was continued for an additional 2.5 hr. The catalyst was removed and the filtrate evaporated at room temperature to give oily O-*t*-butyl-L-threonine *t*-butyl ester (9.98 g, 99.5%) (lit.[6,12,13] pale yellow oil), R_f 0.85.

N-Benzyloxycarbonyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (IV).—Triethylamine (6.00 ml, 0.043 mole) was added to a stirred suspension of N-benzyloxycarbonyl-L-asparagine (11.49 g, 0.043 mole) in nitromethane (100 ml), held in an ice bath at 0°. Within a few minutes the solution became clear, 2-ethyl-5-phenyloxazolium 3′-sulfonate (reagent K) (10.90 g, 0.043 mole) was added, the ice bath was removed, and the mixture was allowed to warm to room temperature. The solution cleared within 15 min, then was cooled to 0° in the ice bath, and O-*t*-butyl-L-threonine *t*-butyl ester (9.98 g, 0.043 mole) in nitromethane (80 ml) was added. After stirring for 5 min at 0°, the ice bath was withdrawn and the reaction allowed to proceed at room temperature for 60 hr. The solvent was evaporated and the residual oil was partitioned between ethyl acetate and water. The organic phase was washed in turn with 1% citric acid solution, water, saturated sodium bicarbonate solution, and water. After drying ($MgSO_4$) and filtering, the solvent was evaporated to afford N-benzyloxycarbonyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (15.36 g, 74.6%): mp 45–49° [lit.[3,6] 47–49° and 118–119°, 120–121° (the higher melting point value is obtained when the product is crystallized from benzene–petroleum ether)]; R_f 0.55; ν_{max}^{KBr} 2970 (CH), 1720 (C=O), 1660 (CONH), 1362 and 1389 (*t*-butyl), and 695 (Ph) cm^{-1}; $\lambda_{max}^{ethanol}$ 253, 258, 264, 267 mμ (ε 280, 364, 413, 417).

L-Asparaginyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (V).—N-Benzyloxycarbonyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (15.36 g, 0.032 mole) was dissolved in methanol (200 ml) containing 10% palladium-on-carbon catalyst (2.27 g) and hydrogenated for 4 hr. Fresh catalyst (0.36 g) suspended in ethyl acetate (20 ml) was added and hydrogenation was continued for a further 4 hr. The catalyst was filtered off and the filtrate was evaporated to an oil, which gradually solidified on standing under petroleum ether. The crude L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester was recrystallized from ethanol–ether–petroleum ether to yield small needles (7.90 g, 71.8%): mp 150–152°; $[\alpha]^{25}D$ +3.3° (*c* 1.27, dimethylformamide) [lit.[3,6] 149°, 150–150.5°, $[\alpha]^{21}D$ +3.0° (*c* 1.27, dimethylformamide), $[\alpha]^{20}D$ +3.0° (*c* 1.8, dimethylformamide)]; R_f 0.16; ν_{max}^{KBr} 3420 (NH), 2970 (CH), 1720 (C=O), 1650 broad (CONH), 1362 and 1390 (*t*-butyl) cm^{-1}.

Anal. Calcd for $C_{16}H_{31}N_3O_5$: C, 55.63; H, 9.05; N, 12.16. Found: C, 55.33; H, 8.91; N, 12.10.

(11) Melting points are uncorrected. Microanalyses were provided by Messrs. Erich H. Meier and J. Consul, Microanalytical Laboratory, Stanford University. The infrared, optical rotations, and ultraviolet measurements were obtained by Mrs. Linda D. Carroll. Thin layer chromatography employed baked silica gel G as the support, methanol–chloroform (1:9) as the solvent, and iodine for detection purposes. Evaporations were performed under reduced pressure, using a rotary evaporator.

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N-Benzyloxycarbonyl-L-methionine 2,4,5-Trichlorophenyl Ester (VI).—N,N′-Dicyclohexylcarbodiimide (6.49 g, 0.0315 mole) in ethyl acetate (20 ml) was added to a stirred solution of N-benzyloxycarbonyl-L-methionine (8.50 g, 0.030 mole) and 2,4,5-trichlorophenol (5.93 g, 0.030 mole) in ethyl acetate (80 ml), previously cooled to 0°. After 2 hr the cooling bath was removed and the reaction was allowed to continue overnight at room temperature. The urea was filtered off and washed with a little ethyl acetate (6.72 g, 100%). The combined filtrates were evaporated to afford N-benzyloxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester, which was crystallized from ethyl acetate–petroleum ether as long, thin, white needles (12.59 g, 90.6%): mp 112.5–113.5°; $[\alpha]^{29}D$ −31.4° (*c* 1.02, dimethylformamide); ν_{max}^{KBr} 3330 (NH), 1782 and 1685 (C=O), 700 (Ph) cm^{-1}; $\lambda_{max}^{ethanol}$ 227 sh, 279, 288 mμ (ε 12,600, 1020, 1115).

Anal. Calcd for $C_{19}H_{18}Cl_3NO_4S$: C, 49.31; H, 3.92; Cl, 22.99; N, 3.03. Found: C, 49.31; H, 3.98; Cl, 22.68; N, 3.20.

N-Benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (VII). A. Active Ester Coupling.—A solution of L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (3.46 g, 0.010 mole) and N-benzyloxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester (4.65 g, 0.010 mole) in dimethylformamide (30 ml) was allowed to stand at room temperature for 1 day. The solvent was evaporated and the residual oil was obtained as a solid from ethanol–ether–petroleum ether, mp 104–107°. Pure N-benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester was procured by crystallization from aqueous methanol as tiny needles (5.26 g, 85.4%): mp 147–147.5°; $[\alpha]^{24}D$ −22.8° (*c* 1.01, methanol); R_f 0.53; ν_{max}^{KBr} 3420 (NH), 2975 (CH), 1720 (C=O), 1660 broad (CONH), 1367 and 1391 (*t*-butyl), and 697 (Ph) cm^{-1}.

Anal. Calcd for $C_{29}H_{46}N_4O_8S$: C, 57.05; H, 7.60; N, 9.18. Found: C, 56.78; H, 7.64; N, 9.34.

B. Reagent K Coupling.—A solution of N-benzyloxycarbonyl-L-methionine (0.284 g, 0.0010 mole) and triethylamine (0.14 ml, 0.0010 mole) in nitromethane (10 ml) was mixed with a stirred suspension of reagent K (0.253 g, 0.0010 mole) in nitromethane (5 ml). The reaction cleared within 1 hr at room temperature, then L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (0.347 g, 0.0010 mole) was added, and the solution allowed to stand overnight. The solvent was evaporated and the residual oil was partitioned between ethyl acetate and water. The organic phase was washed in turn with dilute citric acid, water, dilute sodium bicarbonate solution, and water. After drying ($MgSO_4$) and filtering, the solvent was evaporated to leave a colorless gel. The product was crystallized from aqueous methanol to yield a white solid (0.320 g, 52.4%): mp 146–148°; $[\alpha]^{28}D$ −20.5° (*c* 1.00, methanol; R_f 0.53; identical infrared spectrum with an authentic sample.

L-Methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (VIII).—N-benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (0.203 g, 0.00033 mole) was dissolved in methanol containing 30% palladium-on-carbon catalyst (0.254 g). After hydrogenating for 6 hr, fresh catalyst (0.117 g) suspended in ethyl acetate (5 ml) was added and the reaction continued for a further 15 hr. The solvent was removed and the oily product was partitioned between 1% citric acid and ethyl acetate. The aqueous phase was made alkaline with sodium bicarbonate and extracted with ethyl acetate. The organic phase was dried (Na_2SO_4), filtered, and evaporated to give a colorless oil (0.139 g, 88.4%) [lit.[3,6] 137° (phthaloylhydrazide complex), amorphous substance].

Anal. Calcd for $C_{21}H_{40}N_4O_6S$: C, 52.92; H, 8.46; S, 6.70. Found: C, 52.41; H, 8.10; S, 6.41.

N-Benzyloxycarbonyl-L-methionyl Sulfoxide L-Asparaginyl-O-*t*-butyl-L-threonine *t*-Butyl Ester (IX).—A solution of N-benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (0.307 g, 0.00050 mole) in ethanol (1.2 ml, prepared by warming) was treated with 30% hydrogen peroxide solution (0.07 ml, 0.000625 mole) and allowed to stand at room temperature for 3 days. The solution was evaporated to yield N-benzyloxycarbonyl-L-methionyl sulfoxide L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (0.305 g, 96.6%). The analytical sample was crystallized from ethyl acetate: mp 196–197°; $[\alpha]^{28}D$ −29.7° (*c* 1.01, methanol); R_f 0.25; ν_{max}^{KBr} 3400 broad (NH), 2972 (CH), 1720 (C=O), 1670 broad (CONH), 1367 and 1390 (*t*-butyl), 1030 broad (S=O), and 695 (Ph) cm^{-1}.

Anal. Calcd for $C_{29}H_{46}N_4O_9S$: C, 55.58; H, 7.40; N, 8.94; S, 5.10. Found: C, 55.39; H, 7.18; N, 8.95; S, 5.26.

Thioglycolic Acid Reduction.—A soution of N-benzyloxycarbonyl-L-methionyl sulfoxide L-asparaginyl-O-*t*-butyl-L-threonine *t*-butyl ester (0.095 g, 0.0015 mole) in dioxane (2 ml) and water (1 ml) was mixed with thioglycolic acid (0.105 ml, 5 equiv) and kept at 50° under a nitrogen atmosphere for 8 hr. The solution was evaporated, and the oily residue crystallized from aqueous methanol to produce compound VIII: mp 146–148°; $[\alpha]^{26}D$ −21.6° (*c* 1.00, methanol); R_f 0.53; identical infrared spectrum with an authentic sample.

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Amino Acids and Peptides. VI.[1] Synthesis of a Heptapeptide Sequence (A_{20}–A_{26}) of Glucagon

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During the past several years, various approaches to the synthesis of the A_{20}–A_{26} segment of the hyperglycemic hormone glucagon[2] have been elaborated in some detail. For example, N-*t*-butyloxycarbonyl-L-glutaminyl-L-asparaginyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophan hydrazide,[3] a hexapeptide that spans the A_{20}–A_{25} region, was formed by an azide reaction of N-*t*-butyloxycarbonyl-L-glutaminyl-L-asparaginyl-L-phenylalanine hydrazide and L-valyl-L-glutaminyl-L-tryptophan benzyloxycarbonylhydrazide. The related A_{20}–A_{23} tetrapeptide N-trifluoroacetyl-L-glutaminyl-β-*t*-butyl-L-aspartyl-L-phenylalanyl-L-valine hydrazide was made by a N,N′-dicyclohexylcarbodiimide[4] coupling involving N-trifluoroacetyl-β-*t*-butyl-L-aspartic acid and L-phenylalanyl-L-valine benzyloxycarbonylhydrazide.[5] The tripeptide N-trifluoroacetyl-L-glutaminyl-L-tryptophanyl-L-leucine hydrazide, which covers the short A_{24}–A_{26} sequence, was obtained through a similar N,N′-dicyclohexylcarbodiimide condensation between N-trifluoroacetyl-L-glutamine and L-tryptophanyl-L-leucine *t*-butyloxycarbonylhydrazide.[6] Most recently, the A_{22}–A_{26} area has been encompassed by the two related pentapeptides, N-phthalyl-L-phenylalanyl-valyl-L-glutaminyl-L-tryptophanyl-L-leucine hydrazide and N-phthalyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine. The former compound was prepared by an azide reaction utilizing N-phthalyl-L-phenylalanyl-L-valine hydrazide and L-glutaminyl-L-tryptophanyl-L-leucine butyloxycarbonylhydrazide, while the latter product was constructed by a stepwise prolongation that began with L-leucine *t*-butyl ester.[7]

In continuation of our earlier work on protected subunits of glucagon, there is described here a preparation of a heptapeptide that constitutes the complete A_{20}–A_{26} sequence, N-benzyloxycarbonyl-L-glutaminyl-β-*t*-butyl-L-aspartyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester (I). The synthesis began with a N,N′-dicyclohexylcarbodiimide condensation of N-benzyloxycarbonyl-L-tryptophan[8] and L-leucine methyl ester[9] to give the corresponding dipeptide, N-benzyloxycarbonyl-L-tryptophanyl-L-leucine methyl ester (II). The blocking N-benzyloxycarbonyl group of compound II was removed by hydrogenolysis, and the resulting amine (III) was treated with N-benzyloxycarbonyl-L-glutamine 2,4,5-trichlorophenyl ester[10] to form the tripeptide N-benzyloxycarbonyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester (IV). The polypeptide chain of IV was then progressively lengthened in the same manner through four more amino acid residues by utilizing in turn N-benzyloxy-carbonyl-L-valine 2,4,5-trichlorophenyl ester,[10] N-benzyloxycarbonyl-L-phenylalanine 2,4,5-trichlorophenyl ester,[11] N-benzyloxycarbonyl-β-*t*-butyl-L-aspartic acid *p*-nitrophenyl ester,[12,13] and N-benzyloxycarbonyl-L-glutamine 2,4,5-trichlorophenyl ester.[10] The end product was the desired heptapeptide (I). This sequence compares favorably with the other synthetic procedures mentioned earlier, and has the additional merit of employing pure, crystalline activated ester intermediates, which have been widely used in recent years.[14]

Finally, an alternative route to N-benzyloxycarbonyl-β-*t*-butyl-L-aspartic acid *p*-nitrophenyl ester is described here, in addition to a preparation of the dipeptide N-benzyloxycarbonyl-β-*t*-butyl-L-aspartyl-L-phenylalanine methyl ester (XII). Attention is called to the fact that the β-*t*-butyl blocking group is easily seen in the infrared, with two bands found at about 1390 and 1370 cm^{-1}, the intensity of the latter being much greater.

Experimental Section[15]

N-Benzyloxycarbonyl-L-tryptophanyl-L-leucine Methyl Ester (II).—To a stirred solution of N-benzyloxycarbonyl-L-tryptophan (34.6 g, 0.105 mole)[8] in acetonitrile (400 ml) in an ice bath at −10° was added a freshly prepared solution of L-leucine methyl ester [obtained by treating L-leucine methyl ester hydrochloride (21.5 g, 0.109 mole)[9] with 50% potassium carbonate solution (40 ml) at 0°, extraction of the free ester with ether (three 100-ml portions), and drying the combined organic phases for a short period (Na_2SO_4)], followed by dropwise addition of N,N′-dicyclohexylcarbodiimide (22.7 g, 0.110 mole) in acetonitrile (50 ml). After 8 hr the bath was removed and the mixture was allowed to stand for 36 hr. The N,N′-dicyclohexylurea (21.0

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(15) Melting points are uncorrected. Microanalyses were provided by Messrs. Erich H. Meier and J. Consul, Microanalytical Laboratory, Stanford University. The infrared, optical rotation, and ultraviolet measurements were obtained by Mrs. Linda D. Carroll. Infrared samples were prepared in potassium bromide disks, while ultraviolet spectra were recorded with ethanol as the solvent, unless it is otherwise stated. Thin layer chromatography employed silica gel G as the support, methanol–chloroform (1:9) as the solvent, and iodine for detection purposes. Evaporations were performed under reduced pressure.