THE SYNTHESIS AND TESTING OF SOME ANALOGUES OF PANTOIC ACID AND PANTOTHENIC ACID

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CHESTER ALBERT SCHINK

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APPROVED:

Redacted for Privacy

Associate Professor of Chemistry

In Charge of Major

Redacted for Privacy

Head of Department of Chemistry

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean, Graduate School

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THE SYNTHESIS AND TESTING OF SOME ANALOGUES OF PANTOIC ACID AND PANTOTHENIC ACID

INTRODUCTION

Since the elucidation of the structure of pantothenic acid* (40) many analogues and derivatives of the compound have been prepared. The earlier ones resulted from studies on the structure of the vitamin (25), and from efforts to produce compounds of similar biological activity (3,24,26,29,36,38). However, following the preparation of pantoyl taurine (4,20,34), most syntheses have been aimed at the development of microbiological growth inhibitors related to pantothenic acid. In vitro growth tests with microorganisms have revealed a number of compounds which competitively inhibit the growth-promoting action of pantothenic acid over a wide range of concentrations.

The greater part of the work which has been done in this direction has been in modifying the amino acid moiety of the molecule by means of an amino acid (4,27,33,34), amino ketone (43), amino alcohol (35) or amine (33). The few alterations in the pantoic acid** moiety, on the other hand, have, with but one exception (7), given rise to inactive or very slightly stimulatory substances, when tested on organisms requiring the preformed vitamin.

It was felt that an organism such as Acetobacter, which utilizes pantoic acid as readily as the intact vitamin (32), might be inhibited by compounds resembling this acid, as well as their condensation products with β-alanine. For this reason the synthesis of several analogues of

*"Pantothenic acid" which was coined by Williams (39) is understood to be N-(α,γ-dihydroxy-β,β-dimethylbutyro)-β-aminopropionic acid.
**"Pantoic acid" was suggested by Snell (35) for α,γ-dihydroxy-β,β-dimethylbutyric acid and "pantolactone" for α-hydroxy-β,β-dimethyl-γ-butyrolactone.

pantoic acid and their subsequent coupling with β-alanine was undertaken. Two compounds have been found which effectively inhibit the coupling of pantoic acid to β-alanine. Other analogues have been prepared which possess considerable growth-promoting activity. As the work progressed, it appeared possible that a peptide of β-alanine and glutamic acid might exist in microbial cells. To study this new information, the several peptides of these two amino acids were prepared and tested.

EXPERIMENTAL

Synthesis of Compounds

Throughout this work the emphasis has been placed on the final products, therefore many of the reactions have not been investigated fully and the yields are not necessarily the maximum which might be obtained. Racemic mixtures were not resolved into the optical isomers.

dl-α-Hydroxy-β,β-dimethylbutyric acid. -- Pinacol hydrate was prepared in the usual manner (12,p.459). A yield of 394 g. (53%) was obtained from 80 g. of magnesium turnings. Three hundred and seventy-five grams of pinacol hydrate were dehydrated with sulfuric acid, following the customary procedure (12,p.462), to give 78 g. (47%) of pinacolone.

Forty grams (1.65 moles) of pinacolone in 250 g. of a 20% sodium hydroxide solution were oxidized by adding dropwise 2400 ml. of a 5% potassium permanganate solution (0.76 mole) in the cold according to the method of Glücksmann (14). The reaction mixture was filtered, neutralized, and evaporated to dryness. The residue was dissolved in 300 ml. of water and treated with 500 g. of a 4% sodium-mercury amalgam. The aqueous layer was acidified with dilute sulfuric acid and then extracted with ether. About one liter of water was added to the residue from the distillation of the ether. The solution was distilled to a volume of about 100 ml. and then extracted with ether. The ether solution was concentrated and the concentrated solution was placed in a vacuum desiccator over sulfuric acid; 11.4 g. (21.6%) of white crystals were obtained, m.p. 87.0-87.5°C.

Sodium N-(α-hydroxy-β,β-dimethylbutyro)-β-alanine. -- The coupling of dl-α-hydroxy-β,β-dimethylbutyric acid and β-alanine was carried out according to the procedure of Parke and Lawson (28). To 18 ml. of refluxing isopropyl alcohol was added 0.52 g. (0.023 mole) of sodium through the condenser. After complete solution, 2.00 g. (0.022 mole) of β-alanine were added and the mixture was allowed to reflux for 15 minutes, then 2.97 g. (0.022 mole) of dl-α-hydroxy-β,β-dimethylbutyric acid were added and the refluxing was continued. Within 15 minutes a white curdy precipitate appeared. After the mixture had refluxed for three hours, 15 ml. of isopropyl alcohol were added and the mixture was allowed to cool in the refrigerator. The cold mixture was filtered and the residue washed with cold isopropyl alcohol. Yield 4.5 g. (82%). Analysis calculated for $C_9H_{16}O_4NNa$: N, 6.21; Na, 10.20. Found: N, 6.09; Na, 10.35.

Methallyl cyanide (3 Methyl-3-butenenitrile). The procedure described in "Organic Syntheses" (12,p.46) for allyl cyanide was found to be applicable to the preparation of methallyl cyanide. One hundred and ten grams (1.22 moles) of freshly distilled methallyl chloride and 112 g. (0.62 mole) dried cuprous choloride were heated on a water bath in a 500 ml. round bottom flask equipped with a mercury seal stirrer and a bulb type reflux condenser. A heating period of two to five hours was necessary to start the reaction. After the reaction had subsided, the nitrile was distilled from the reaction mixture by heating on an oil bath; stirring being continued throughout the distillation. The crude product was redistilled with the fraction boiling at 134.5-136°C. being

collected*. Yield 67.3 g. (68.5%).

dl-β-Hydroxy-β-methyl-γ-butyrolactone. -- Peracetic acid was prepared by adding 56 g. (0.50 mole) of 30% hydrogen peroxide to 115 ml. of glacial acetic acid and warming the mixture at 80°C. for 45 minutes. Twenty grams (0.25 mole) of methallyl cyanide were added to the cooled solution. This solution was allowed to stand at room temperature for one week.

The volume of the solution was reduced to about one fourth by distillation in vacuo. Twenty-five milliliters of 6N HCl were added to the residue and the mixture was refluxed for one hour, after which it was distilled at reduced pressure to a small volume. The pasty residue was extracted with ether. The oil obtained upon evaporation of the ether was dissolved in 25 ml. of water and 25 ml. of concentrated HCl were added. The mixture was refluxed for two hours. The HCl solution was distilled in vacuo until a sticky solid remained. This residue was extracted with several portions of acetone. The acetone solution was dried over sodium sulfate, after which the acetone was evaporated, leaving a brown viscous oil. The oil was partially purified by repeated solution in ether-acetone mixtures, removal of the insoluble portion, and evaporation of the solvent.

Sodium N-(β,γ-dihydroxy-β-methylbutyro)-β-alanine. -- Five grams of partially purified dl-β-hydroxy-β-methyl-γ-butyrolactone were coupled with β-alanine as previously described. The coupled

*Tamele et al (37) prepared this compound using nitrobenzene as a solvent for the reaction. They gave 136.2-136.4°C. as the boiling point with a yield of 58%.

product was purified by repeated solution in methanol and precipitation in a large excess of dry acetone. The product was a light brown, amorphous, deliquescent solid which could not be decolorized by charcoal. Yield, (20%). Analysis calculated for $C_8H_{14}O_5N$ Na: N, 6.16; Na, 10.12. Found: N, 6.33; Na, 10.32.

β,β-Dimethyl-γ-butyrolactone*. -- β,β-Dimethylglutaric acid, prepared from malonic ester and mesityl oxide according to the method of Komppa (19), was oxidized with iodine to give the desired β,β-dimethyl-γ-butyrolactone (41), m.p. 56°C.

Sodium N-(γ-hydroxy-β,β-dimethylbutyro)-β-alanine. -- The product was prepared as described above using 0.043 g. (0.0019 mole) sodium, 2 ml. isopropyl alcohol, 0.166 g. (0.0019 mole) β-alanine and 0.213 g. (0.0019 mole) β,β-dimethyl-γ-butyrolactone. Yield, 245 mg. (58%). Analysis calculated for $C_9H_{16}O_4N$ Na: N, 6.21. Found: N, 5.90.

Sodium N-(α-hydroxy-β,β-dimethylbutyro)-taurine. -- This condensation was carried out as described above using 0.35 g. (0.015 mole) sodium, 15 ml. isopropyl alcohol, 1.89 g. (0.015 mole) taurine, and 2.00 g. (0.015 mole) dl-α-hydroxy-β,β-dimethylbutyric acid. The product was purified by recrystallizing from absolute ethanol; m.p. 190-195°C. Yield about (70%). Analysis calculated for $C_8H_{16}O_5NSNa$: N, 5.36. Found: N, 5.21.

Diethyl-N-(α,γ-dihydroxy-β,β-dimethylbutyro)-l(+) glutamate. -- One gram (0.0049 mole) of diethyl-l(+) glutamate (prepared from l-(+) glutamic acid, absolute ethanol and dry HCl (9)) and 0.64 g. (0.0049 mole) of dl-pantolactone were heated together in an oven at 120°C. for

*This compound was prepared by Mr. L. W. Clark.

3.5 hours. The product was a light yellow viscous oil which contained no amino nitrogen, as shown by a Van Slyke determination*, therefore complete coupling was assumed. Yield (100%).

Ethyl-N-methylpantothenate. (ethyl-N-methyl-N-(α,γ-dihydroxy-β,β-dimethylbutyro)-β-alanine). — Nine-tenths gram (0.0069 mole) of pantolactone and 0.92 g. (0.0069 mole) of ethyl-β-methylamino-propionate (ethyl ester of N-methyl-β-alanine, prepared from bromo-propionic acid and methylamine (21)) were heated together at 120°C. for five hours. The light yellow, viscous oil had a slight odor resembling acylic acid or its ester. When a portion of the product was treated with an excess of dry ether, a white precipitate appeared which was removed by centrifuging. Evaporation of the ether solution yielded a clear light yellow oil. Yield (89%). Analysis calculated for $C_{12}H_{23}O_5N$: N, 5.36. Found: N, 4.6. (The low N content may likely be due to the presence of unreacted pantolactone; the compound was 4% as active as pantoic acid for A. suboxydans (Table III).)

Pantoyl taurine (Sodium-N-(α,γ-dihydroxy-β,β-dimethylbutyro)-taurine). — This compound was prepared according to the method of Snell (34) by fusing 1.77 g. (0.014 mole) of dl-α-hydroxy-β,β-dimethyl-γ-butyrolactone and 2.00 g. (0.014 mole) of the sodium salt of taurine. The product was purified by dissolving it in about 50 ml. of absolute ethanol, filtering, and precipitating by pouring the solution into a large excess (350 ml.) of dry ether. The flocculent precipitate was centrifuged and dried in vacuum. Yield 2.13 g. (56%).

*This determination was made by Mr. E. C. Bubl.

Analysis calculated for $C_8H_{16}O_6NSNa$: N, 5.06. Found: N, 4.86.

N-Pantoyl-n-butylamine (N-(α,γ-dihydroxy-β,β-dimethylbutyro)-n-butylamine). — This compound was prepared as described by Shive and Snell (33), using dl-α-hydroxy-β,β-dimethyl-γ-butyrolactone and n-butylamine. m.p. 52°C. Yield (29%) of purified product.

β-Alanyl-l(+)-glutamic acid (β-aminopropionyl-l(+)-glutamic acid). β-Alanyl-l(+)-glutamic acid was prepared according to the Fischer synthesis (10). β-Bromopropionyl chloride was prepared by refluxing 15 g. (0.098 mole) of β-bromopropionic acid and 70 ml. (0.97 mole) of thionyl chloride for three hours. The thionyl chloride was distilled at atmospheric pressure and the residue under reduced pressure; 11.7 g. (70%) being collected at 52-58°C./15 mm*.

Ten grams (0.068 mole) of l(+)-glutamic acid were dissolved in 136 ml. of N NaOH (0.136 mole) and the solution cooled in an ice-salt bath. Twelve grams (0.070 mole) of cold β-bromopropionyl chloride and 136 ml. of cold N NaOH (0.136 mole) were added in small portions with vigorous shaking over the course of an hour. When the odor of the acid chloride had disappeared (15 min.), 200 ml. of N HCl were added. The solution was evaporated to dryness under reduced pressure. The residue was extracted with hot ethyl acetate; petroleum ether was added to the ethyl acetate solution, causing an oil to settle out. This oil was separated, dissolved in ethyl acetate and reprecipitated with petroleum ether. The oil was then aspirated to remove any solvents.

Fourteen grams of the crude β-bromopropionyl-l(+)-glutamic acid

*Hamilton and Simpson (15), using a different method of preparation of this compound, give the boiling point as 65-70°C./25-30 mm.

were treated with 100 ml. of 28% NH_4OH. The solution was allowed to stand for three days at room temperature and was then evaporated on a water bath to a syrupy residue. The residue was dissolved in 5-10 ml. of a water-methanol mixture and precipitated by adding the solution, with stirring, to a large excess (300 ml.) of absolute ethanol. This procedure was repeated, and a white deliquescent solid was obtained. Yield approximately (70%). Analysis calculated for $C_8H_{14}O_5N_2$: N, 12.84. Found: N, 13.11.

N-Carbobenzoxy-l(+)-glutamic anyhdride. — This compound, as well as several others described below, were employed as intermediates in the preparation of peptides of glutamic acid and β-alanine. They have been prepared following the general methods given by Bergman (5,6) for the use of carbobenzoxychloride in the general preparation of peptides.

Carbobenzoxychloride was prepared by adding freshly distilled benzyl alcohol to a 20% phosgene solution in toluene. After the mixture had been allowed to stand at room temperature for three hours, the toluene was evaporated under reduced pressure, yielding about 90% of the crude product. The carbobenzoxychloride was reacted with l(+)-glutamic acid by shaking a cold aqueous suspension of the two materials in the presence of magnesium oxide. The acidified solution was extracted with ethyl acetate. Evaporation of the ethyl acetate under reduced pressure yielded 87% of solid N-carbobenzoxy-l(+)-glutamic acid, m.p. 118-120°C. Warming the above compound with acetic anhydride at 100°C. for five minutes followed by evaporation of the solvent gave

97% of N-carbobenzoxy-l(+)-glutamic anhydride.

N-carbobenzoxy-l(+)-glutamyl-β-alanine ethyl ester. -- To 6.8 g. (0.026 mole) of N-carbobenzoxy-l(+)-glutamic anhydride in 25 ml. of dry chloroform were added 6.1 g. (0.052 mole) of ethyl-β-amino-propionate (prepared from β-alanine in absolute ethanol saturated with dry HCl; the hydrochloride having been removed with the calculated amount of sodium-methoxide). Considerable heat was evolved when the amino acid ester was added and it was necessary to cool the reaction. After the reaction mixture had been allowed to stand at room temperature for five hours it was washed with two 50 ml. portions of 3N HCl and dried. The chloroform was evaporated under reduced pressure and the oily residue dissolved in a minimum amount of hot absolute ethanol. The pasty solid which settled out on cooling the alcohol solution in the refrigerator was removed by filtration and triturated in an ethanol-ether-petroleum ether mixture, filtered and dried; weight of product 5.8 g. (59%), m.p. 94-96°C. Analysis calculated for $C_{18}H_{24}O_7N_2$: N, 7.36. Found: N, 7.13.

N-carbobenzoxy-l(+)-glutamyl-β-alanine. -- Five and six-tenths grams (0.015 mole) of the previous compound were dissolved in 30 ml. of N NaOH (0.030 mole) and the mixture was allowed to stand at room temperature for 30 minutes. When the solution was made acid to congo red with 6 N HCl, an oily layer appeared. Crystallization was induced by dissolving the oil in a small amount of absolute ethanol and adding a 1:1 ether-petroleum ether mixture and cooling in the refrigerator. Yield (58%), m.p. 152-153°C., N.E. 178; theory, 176.

1(+)-Glutamyl-β-alanine. — A portion of the above compound was dissolved in methanol containing a few drops of glacial acetic acid. To this was added about 10% of a catalyst which contained 10% palladium on charcoal. Hydrogenation was effected in the Parr apparatus at a pressure of two atmospheres of hydrogen for one hour. (Hydrogenation at atmospheric pressure resulted only in the recovery of the starting materials.) The residue from the removal of the catalyst and solvents was dissolved in a small amount of water and precipitated by the addition of ethanol. The product was a white hygroscopic solid, m.p. 86-88°C. Analysis calculated for $C_8H_{14}O_5N_2$: N, 12.82. Found: N, 12.75.

β-Methyl-β-bromobutyronitrile (3-bromo-3-methylbutyronitrile). Ten grams (0.12 mole) of methallyl cyanide in 20 ml. of chloroform were treated with an excess of dry hydrogen bromide. The chloroform was evaporated and the residue distilled: the fraction boiling at 95-96°C./42-45 mm. was collected and redistilled. The fraction boiling at 98-100°C./46-50 mm. was collected as the product. It solidified in the condenser so that it was necessary to circulate warm water through the condenser. Eleven grams (55%) of a white waxy solid were collected, m.p. 35-36°C. Analysis calculated for C_5H_8NBr: N, 8.69; Br, 49.6. Found: N, 8.53; Br, 49.4.

2-Bromo-2-methyl-1-propanol. — Thirty milliliters (0.36 mole) of methallyl alcohol, 200 mg. of hydroquinone, and a trace of anhydrous aluminum chloride were placed in an 8 inch pyrex test tube equipped with an inlet tube at the bottom and a reflux condenser. The reaction vessel was cooled in an ice bath. Dry hydrogen bromide was bubbled

through the solution until an excess was present as evidenced by fuming at the top of the condenser. The product separated into two layers, the upper layer being present in the greater amount. The upper layer was aspirated to remove the excess HBr. It was then distilled under reduced pressure, 37.9 g. (68%) being collected at 117-120°C./50-55 mm. A bromine determination was made by titrating an alcoholic solution of the sample with aqueous silver nitrate in the cold. Analysis calculated for C_4H_9OBr: Br, 52.2. Found: Br, 52.4. This product was used in an attempt to synthesize β,β-dimethyl-γ-butyrolactone (See page 15).

Silver pantoate (silver α,γ-dihydroxy-β,β-dimethylbutyrate) (13). -- Two grams (0.015 mole) of dl-α-hydroxy-β,β-dimethyl-γ-butyrolactone were dissolved in 7.5 ml. of 6N NH_4OH, the solution was warmed on the water bath and then aspirated to remove the excess ammonia. A solution of 2.6 g. (0.015 mole) of silver nitrate in 5.0 ml. of water was then added. The resulting solution was evaporated to near dryness under reduced pressure. The precipitate was recrystallized from 95% ethanol yielding 0.91 g. (25%) of purified product in the form of gray crystals which darkened at 125-130°C. and melted at 149-152°C. Analysis calculated for $C_6H_{11}O_4Ag$: Ag, 42.4. Found: Ag, 42.5. This product was used in an attempt to synthesize the ester of pantoic acid and ethyl β-hydroxypropionate (See page 14).

Lead pantoate (lead-α,γ-dihydroxy-β,β-dimethylbutyrate). -- Five grams (0.038 mole) of racemic pantolactone were dissolved in 25 ml. of water and 15 ml. of 6N NH_4OH were added. The mixture was

warmed on the water bath for about thirty minutes and then aspirated to remove the excess ammonia. To this was added a solution of 7.25 g. (0.019 mole) of $Pb(C_2H_3O_2)_2.3H_2O$ in 15 ml. of water. The resulting mixture was evaporated to dryness under reduced pressure, and the residue recrystallized from 95% ethanol giving 7.5 g. (83%) of a white powdery solid. Analysis calculated for $(C_6H_{11}O_4)_2Pb$: Pb, 41.5. Found: Pb, 41.5. This product was used in an attempt to synthesize the ester of pantoic acid and ethyl-β-hydroxypropionate (See page 14).

Attempted halogenation pantoyl lactone. — Various halogenating agents were used in an attempt to replace one or both of the hydroxyl groups of dl-α-hydroxy-β,β-dimethyl-γ-butyrolactone by a halogen atom. The results are summarized in the following table:

TABLE I

THE EFFECT OF HALOGENATING AGENTS UPON PANTOLACTONE

Reagent	Ratio: Reagent / Lactone	Temp. °C.	Time (hrs)	Appearance of Product	%X^-*	Remarks
$SOCl_2$	1.4	25	48	oil	0.7	
$POCl_3$	0.5	25	48	oil	3.0	
$POCl_3$	1.4	107	0.5	black tar	---	
$POCl_3$-PCl_5	1.2	25	16	viscous oil	9.4	
$POCl_3$-PCl_5	0.4	100	2.5	black sticky solid	---	
PBr_3	1.1	25	48	sticky solid	19.6	
PBr_3	1.1	100	2.5	oil	23.0	
PBr_3	1.6	175	1	oil	20.1	
48% HBr	1.3	25	48	oil	0.4	trace of zinc added
48% HBr	1.3	100	0.5	oil	4.9	trace of zinc added
gaseous HBr	---	0	3	oil	33.9	ether as solvent
gaseous HBr	---	0	2	oil	22.6	$CHCl_3$ as solvent

*Theory for 1 Cl^- = 24.4%; for 1 Br^- = 41.5%

In practically every case the product was purified by pouring the reaction mixture (after evaporating the solvents when necessary) into an ice-water mixture (the products with the higher halogen content were insoluble in water), extracting with ether, and distilling the ether. Distillation of the products did not change the halogen content, nor did rebromination of the last two samples above.

Attempted esterification of pantoic acid. — One hundred and forty-two milligrams (0.00056 mole) silver pantoate (See page 12) were dissolved in 55 ml. of 90% ethanol. To this solution was added 100 mg. (0.00055 mole) of ethyl-β-bromopropionate and the mixture was allowed to stand in the dark for 17 days at 25°C. A dark gray precipitate formed, which was filtered off and the filtrate concentrated under reduced pressure. The residue possessed a neutral equivalent of 164. (Theory for the desired product is 122.)

A mixture of 2.0 g. (0.0040 mole) of lead pantoate (See page 12), 100 ml. of n-propanol, and 3.4 g. (0.0019 mole) of ethyl-β-bromopropionate were allowed to stand at 37°C. for 75 days. The mixture was filtered and the filtrate concentrated under reduced pressure leaving 1.0 g. of an oil; 1.89 g. of solid were recovered from the filtration. A saponification equivalent of the liquid gave 174, while the theory for the desired compound is 122, and the starting ester, 181.

Attempts to react the lead pantoate with n-propyliodide in n-propanol resulted only in the recovery of pantolactone and lead iodide.

Treatment of pantolactone with n-propanol, using sulfosalicylic

acid or dry hydrogen chloride as catalysts; or sodium β-hydroxy-propionate by fusion; or n-propylmercaptan at 100°C. resulted in the recovery of the starting materials.

Attempted synthesis of β,β-dimethyl-γ-butyrolactone. — Condensation of 2-bromo-2-methyl-1-propanol with malonic ester was tried following the usual procedures (2, p.18: 12, p.250). Although the sodium malonate disappeared gradually, no product could be isolated which demonstrated the properties expected. Several variations in the treatment were employed, including the use of ether as a solvent, and various periods of refluxing, as well as contact for three days at room temperature.

TESTING

Organisms and Testing. -- The organisms used for testing the various compounds were Acetobacter suboxydans, A. T. C. C. No. 621; Lactobacillus arabinosus 17-5; and Saccharomyces cerevisiae, Gebrüder Mayer strain. (Glutamic acid derivatives were tested with the more sensitive Lash Miller strain.) All tests were performed by using previously published methods (16,31,32). β-Alanine was omitted from the A. suboxydans medium when pantothenic acid was the growth factor.

Results. -- Preliminary experiments showed that with the exception of A. suboxydans, organisms were relatively unaffected by several of the new analogues. Since complete inhibition of growth often requires much higher concentration of the analogues than is needed for partial inhibition, it was decided to express molar analogues: growth factor ratios at 50% inhibition, i.e., where growth is equivalent to that produced by one-half of the growth factor present.

In Table II, the inhibitory action of three analogues of pantoic acid is compared, for three organisms. None of these compounds is able to counteract the growth-promoting effect of pantothenic acid in L. arabinosus, which utilizes only the intact vitamin molecule, or in G. M. yeast, which can synthesize the correct hydroxy acid (pantoic acid) readily. In A. suboxydans, on the other hand, where growth is dependent upon pantoic acid, a competitive inhibition is observed between each antimetabolite and the growth factor. The effective ratios remain fairly constant over wide concentration ranges (data not shown).

The three antimetabolites are most effective when the vitamin moieties are used to promote growth. This is especially true in A.

TABLE II

EFFECT OF PANTOIC ACID ANALOGUES UPON VARIOUS ORGANISMS

Ratio: $\frac{\text{Analogue}}{\text{Growth Factor}}$ at 50% Inhibition

Compound	Lactobacillus arabinosus 17-5	Acetobacter suboxydans		G.M. Yeast	
	GROWTH FACTOR				
Formula	Pantothenic Acid	Pantoic Acid	Pantothenic Acid	β-Alanine	Pantothenic Acid
1) $CH_2OH-C(CH_3)_2-CH_2-COOH$	Inert	10	300	100,000	100,000
2) $CH_3-C(CH_3)_2-CHOH-COOH$	Inert	1000		25,000	Inert
3) $CH_2OH-C(CH_3)OH-CH_2-COOH$	Inert	12,000	100,000	40,000	Inert

suboxydans, where a low $\frac{\text{analogue}}{\text{growth factor}}$ ratio of 10 is observed for β,β-dimethyl-γ-hydroxybutyric acid against pantoic acid. It would appear, therefore, that these hydroxy acids are able to prevent the coupling of β-alanine to pantoic acid which normally occurs in A. suboxydans or yeast when the intact vitamin is not available. As might be expected, yeast is inhibited less by these acids than is A. suboxydans, since pantoic acid is normally produced by yeast. Organisms which synthesize pantothenic acid (35,43) are not in general affected by pantothenic acid analogues.

Table III outlines the effect of analogues of pantothenic acid upon the three test organisms. Compounds 4, 5 and 6 are the β-alanides of Nos. 1, 2 and 3, respectively. Compound 7 is obtained by condensing taurine with No. 2. No. 8 is pantoyl taurine (34), which is included for comparison. No. 9 is N-pantoyl-n-butylamine (33). No. 10 is ethyl-N-methylpantothenate.

The results obtained with Compounds 4, 5 and 6 are in marked contrast to those obtained with 1, 2 and 3. The pantothenic acid analogues are of no value as growth inhibitors, and in fact stimulate growth to some extent in all of the test organisms. The growth stimulation is greatest for yeast, where Compound 5 has virtually the same activity as β-alanine, expressed on a molecular basis. This may be due to hydrolysis by the yeast, since β-alanine would be formed in each case. The relative activities of the three compounds seem to be in accord with their supposed ease of hydrolysis; Compound 5, with a hydroxy group alpha to the amide, should hydrolyze most readily.

The differences between the effect of Compound 7 and pantoyl

TABLE III

EFFECT OF PANTOTHENIC ACID ANALOGUES UPON VARIOUS ORGANISMS

Activity (A) Ratio* and Inhibition (I) Ratio**

Compound	Formula	Lactobacillus arabinosus 17-5	Acetobacter suboxydans		G. M. Yeast	
		GROWTH FACTOR				
		Pantothenic Acid	Pantoic Acid	Pantothenic Acid	β-Alanine	Pantothenic Acid
4)	$CH_2OH-C(CH_3)_2-CH_2CO-NH-CH_2-CH_2-COOH$	$2x10^{-4}$(A)	$5x10^{-4}$(A)	$3x10^{-4}$(A)	$8x10^{-2}$(A)	$3x10^{-2}$(A)
5)	$CH_3-C(CH_3)_2-CHOH-CO-NH-CH_2-CH_2-COOH$	$4x10^{-5}$(A)	$2x10^{-4}$(A)	$1x10^{-4}$(A)	$9x10^{-1}$(A)	$3x10^{-1}$(A)
6)	$CH_2OH-C(CH_3)OH-CH_2-CO-NH-CH_2-CH_2-COOH$	Inert	$1x10^{-4}$(A)		$1.5x10^{-1}$(A)	$3x10^{-2}$(A)
7)	$CH_3-C(CH_3)_2-CHOH-CO-NH-CH_2-CH_2-SO_3H$	Inert	800(I)	2000(I)	Inert	Inert
8)	$CH_2OH-C(CH_3)_2-CHOH-CO-NH-CH_2-CH_2-SO_3H$	1000(I)	$1x10^{-1}$(A)	$1x10^{-1}$(A)	Inert	10,000(I)
9)	$CH_2OH-C(CH_3)_2-CHOH-CO-NH-CH_2-CH_2-CH_2-CH_3$	12,500(I)	$4x10^{-4}$(A)	$4x10^{-4}$(A)	30,000(I)	25,000(I)
10)	$CH_2OH-C(CH_3)_2-CHOH-CO-N(CH_3)-CH_2-CH_2-CO_2-C_2H_5$	$2.5x10^{-6}$(A)	$4x10^{-2}$(A)		Inert	Inert

*Activity (A) ratio= Ratio: $\frac{\text{Growth Factors}}{\text{Analogues}}$ required to produce half-maximum growth

All compounds showing activity were without inhibition at any concentration.

**Inhibition (I) ratio= Ratio: $\frac{\text{Analogues}}{\text{Growth Factors}}$ at 50% inhibition.

taurine are striking. The removal of the γ-hydroxy group changes this potent inhibitor of L. arabinosus and yeast growth into a substance which is completely inert. Conversely, whereas pantoyl taurine promotes the growth of A. suboxydans, this desoxy compound is a good inhibitor. The last observations may be due to the ability of the organism to hydrolyze pantoyl taurine to pantoic acid. A similar hydrolysis of Compound 7 would yield Compound 2, which was shown in Table II to inhibit growth.

Although in our hands Compound 9 is a poorer inhibitor of L. arabinosus growth than previously reported (33), it does compete with pantothenic acid in this organism. It is also effective in yeast against either the vitamin or β-alanine. Its stimulatory action on A. suboxydans is probably due to pantoic acid, which may be present either as a contaminant in the preparation or as a result of hydrolysis of the analogue by the organism. N-methylpantothenic acid, Compound 10, is also seen to be inert.

It is interesting to note that A. suboxydans apparently does not hydrolyze Compounds 4, 5 and 6, for the cleavage products (Compounds 1, 2 and 3, respectively), would be inhibitors.

Table IV shows the results of glutamic acid derivatives with the test organisms. In Compound 11, the diethyl ester of l(+)-glutamic acid replaces the β-alanine moiety of pantothenic acid. Compound 12 is β-alanyl-l(+)-glutamic acid; and Compound 13 is l(+)-glutamyl-β-alanine.

TABLE IV

EFFECT OF GLUTAMIC ACID ANALOGUES UPON VARIOUS ORGANISMS

Activity (A) Ratio* and Inhibition (I) Ratio**

Compound	Formula	Lactobacillus arabinosus 17-5	Acetobacter suboxydans		L. M. Yeast	
		GROWTH FACTOR				
		Pantothenic Acid	Pantoic Acid	Pantothenic Acid	β-Alanine	Pantothenic Acid
11)	$CH_2OH-C(CH_3)_2-CHOH-CO-NH-CH(CO_2C_2H_5)-CH_2-CH_2CO_2C_2H_5$	5000(I)	2.5×10^{-2}(A)		Inert	Inert
12)	$NH_2-CH_2-CH_2-CO-NH-CH(COOH)-CH_2-CH_2-COOH$	Inert	2.5×10^{-5}(A)		Inert	Inert
13)	$NH_2-CH(CO-NH-CH_2-CH_2-COOH)-CH_2-CH_2-COOH$	Inert	1×10^{-2}(A)		2.5×10^{-2}(A)	3×10^{-3}(A)

*Activity (A) ratio = Ratio: $\frac{\text{Growth Factors}}{\text{Analogues}}$ required to produce half-maximum growth

All compounds showing activity were without inhibition at any concentration.

**Inhibition (I) ratio = Ratio: $\frac{\text{Analogues}}{\text{Growth Factors}}$ at 50% inhibition.

DISCUSSION

The condensation of 2-bromo-2-methylpropanol-1 (a hydroxy-tert-butylbromide) with sodium malonic ester was not successful under any of the conditions tried. Abderhalden and Rossner (1) claim to have condensed tert-butylbromide with sodium malonic ester by allowing the mixture to stand at room temperature for three days, However, Homeyer et al (17) state that due to its extreme unreactively tert-butylbromide does not react satisfactorily with malonic ester, acetoacetic ester or in the Grignard reaction.

2-chloro-2-methylpropanol-1 has been prepared by Michael and Leighton (23), who report that it decomposes readily into isobutyraldehyde in the presence of sodium carbonate, potassium acetate or water. Garzino (11), who prepared 1-bromo-2-methylpropanol-2, claimed that this compound also readily decomposed into isobutyraldehyde on boiling.

The inability to obtain the desired product from the above reaction is no doubt mainly due to the instability of the bromopropanol.

The several attempts to replace one or both of hydroxy groups of pantolactone by various widely used reagents always resulted in products with considerably less than the theoretical halogen content. The best results were obtained with phosphorus tribromide and dry hydrogen bromide as shown in Table I, p. 13 . The fact that a higher bromine content of the product could not be obtained by solvent fractionation or vacuum distillation suggests the possibility of some type of compound formation other than that expected.

The failure of pantolactone or the salts of pantoic acid to esterify can no doubt be attributed to the extreme readiness with which gamma hydroxy acids lactonize and the stability of the resulting lactone. Although lead iodide was formed from lead pantoate and n-propyl-iodide, the rate of lactonization of the pantoyl group apparently is far greater than the formation of the ester. Thus n-propanol must be formed from the propyl iodide.

With each organism studied, growth has been influenced in three ways by the various analogues. As pointed out previously (35), some are inert; others possess vitamin activity; while still others appear to compete with the growth factor for attachment within the cell, presumably at the surface of an enzyme.

If the action of different analogues is viewed in the light of the Woods-Fildes theory, it must be assumed that inert compounds are incapable of attachment within the cell, at least in the normal manner. Inert analogues of pantothenic acid are seen to differ from the vitamin in the pantoic acid portion of the molecule, and in the substitution of a methyl group for a hydrogen atom on the amide nitrogen of pantothenic acid. This suggests that the vitamin is normally attached through the pantoic acid portion, since changes about the carboxyl group of β-alanine or in the configuration of the chain of the β-alanine structure seldom, if ever, produce inert analogues. Both hydroxy groups appear important; the removal of either one greatly reduces the activity, and further changes, as in Compound 6, remove the activity altogether. Similar losses in activity have been

recorded by other workers (3,27,34,44), although Barnett and Robinson (3,4) noted some inhibition of growth of E. coli with Compound 4 and with the β-alanides of γ-hydroxybutyric and γ-hydroxy valeric acid. However, the effects were not reversed by pantothenic acid, and it would seem that these analogues do not directly involve the utilization of the vitamin.

Complete loss of activity when the amide hydrogen is substituted by a methyl group (Compound 10) is also indicative of attachment through the nitrogen atom either to a protein or other compound with which the vitamin may normally be conjugated. Unpublished data by King and Cheldelin (18) suggest that pantothenic acid may normally be conjugated with glutamic acid in microbial cells. In view of the present results with Compound 10, it appears possible that this attachment may take place through the amide group.

The behavior of stimulatory and inhibitory analogues is also compatible with the assumption that they are attached through the hydroxy acid moiety, although the picture is somewhat complicated for A. suboxydans and yeast wherever hydrolysis may produce the proper growth factor. For example, a weakend or restricted attachment of Compound 4, 5 or 6 in L. arabinosus or A. suboxydans would still permit the β-alanine structure to engage in metabolic activities. Likewise, pantoyl taurine and other pantoyl amides have the correct configuration in the hydroxy acid moiety, and should attach to the enzyme; but here the alteration or removal of the carboxyl group presumably prevents the analogue from entering into further reactions in

the cell.

One of the best lines of evidence for attachment of pantothenic acid through the pantoic acid moiety in yeast comes from the behavior of pantoyl taurine. Although this compound effectively inhibits growth produced by pantothenic acid, it has been shown (31,34) that it has no influence on growth when β-alanine is supplied. If both the growth factor and the analogue were attached by means of the carboxyl group (or the β-amino group), competition should be found in any case. However, if we regard pantoic acid (produced by the cells) as being combined with the enzyme, β-alanine would be free to couple without interference from pantoyl taurine.

Inhibition of microbial growth may in some cases be due to prevention of proper attachment of the vitamin to the enzyme. This is probably true of pyridine-3-sulfonic acid (22), 3-acetyl pyridine (42) or lumiflavin (30), as well as the β-alanide of α, γ-dihydroxy-β,β-dimethyl valeric acid (7). So far as we have been able to determine, the latter is the only inhibitory analogue of pantothenic acid which exhibits alterations in the pantoic acid moiety.

It is not yet possible to form a detailed picture of the combination of pantothenic acid to cellular enzymes. Organisms differ widely in their resistance to inhibitors, and the different ability of yeast and A. suboxydans to hydrolyze various analogues suggest that the vitamin may be oriented differently toward its respective points of attachment in the two types of cells. Moreover, it is possible that the substituted amide group in pantothenic acid may take part in

some of its reactions. We are attempting to settle this point through the preparation of suitable analogues. The scope of possible reactions which such a group may undergo is limited, however, and in any case it appears difficult on the basis of present evidence to postulate unstable linkages which would permit pantothenic acid to take part *per se* in catalytic reactions. Such speculation favors the existence of catalytically active conjugates of the vitamin, a point which is being pursued at the present time.

SUMMARY

Several new analogues of pantoic acid and pantothenic acid have been prepared. These are: β-hydroxy- β-methyl- γ-butyrolactone, sodium N-(α-hydroxy- β, β-dimethylbutyro)- β-alanine, sodium N-(β, γ dihydroxy- β-methylbutyro)- β-alanine, sodium N-(α-hydroxy- β, β-dimethylbutyro)-taurine, ethyl-N-methyl-N-(α, γ-dihydroxy- β, β-dimethylbutyro)- β-alanine, diethyl-N-(α, γ-dihydroxy- β, β-dimethylbutyro)-l(+)-glutamate, β-alanyl-l(+)-glutamic acid, and l(+)-glutamyl- β-alanine. The above compounds, together with several others previously described, were tested for their growth effects upon A. suboxydans, L. arabinosus, and S. cerevisiae.

3-Bromo-3-methylbutyronitrile and 2-bromo-2-methylpropanol-1 were prepared and used as intermediates in other syntheses.

Three analogues of pantoic acid were observed to competitively inhibit the coupling of β-alanine to pantoic acid in A. suboxydans.

Analogues of pantothenic acid possessing alterations in the β-alanine structure were found to be generally good growth inhibitors. Changes in the pantoic acid moiety produced slightly stimulatory or inert compounds.

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