to stand at room temperature for 24 hours. It was then poured into 45 l. of ice-water and acetic acid was slowly added to decompose excess reagent until no further effervescence was observed. The precipitate was collected and washed well with dilute hydrochloric acid, water, sodium bicarbonate solution and water. The dried product was crystallized from ethyl acetate to yield 325 g. (65%) of pregnane-3α,17α-diol-20-one (XVI) with m.p. 206–211°, $[\alpha]^{20}D$ +56° (EtOH), which was suitable for transformation[22] to "substance S." On further crystallization pure XVI was obtained with m.p. 212–214°, $[\alpha]^{20}D$ +59° (EtOH), $\lambda_{max}^{CHCl_3}$ 1700 cm.$^{-1}$ and free hydroxyl band, identified by direct comparison with an authentic specimen (reported[20] m.p. 213–214°, $[\alpha]^{25}D$ +63° (EtOH)). The mother liquors, through chromatography, could be made to yield another 25 g. of XVI with m.p. 205–210° (total yield 70%). It was however more economical to reoxidize them with N-bromoacetamide in pyridine solution (*cf.* footnote 20), when *ca.* 15% of the original diketone XV were recovered.

When the amount of water in the above described experiment was decreased, the rate of reduction was considerably reduced.

MEXICO CITY 17, D. F.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE SCIENCE RESEARCH INSTITUTE, OREGON STATE COLLEGE]

β-Aletheine[1] and Pantetheine[2]

BY TSOO E. KING, CHARLES J. STEWART AND VERNON H. CHELDELIN

RECEIVED SEPTEMBER 15, 1952

β-Aletheine (N-(β-alanyl)-2-aminoethyl mercaptan) was prepared by condensation of carbobenzoxy-β-alanyl chloride with β-aminoethyl mercaptan, reduction and isolation of the crystalline oxalate. β-Aletheine was then condensed with pantoyl lactone to produce pantetheine of high purity in good yields. β-Aletheine could not replace β-alanine for growth of yeast in a pantothenic acid-free medium, nor was it inhibitory. The pantetheine made by the present method possessed about 20,000 LBF units per mg. when assayed by *L. helveticus*, and upon enzyme digestion yielded at least 95% of the theoretical amount of pantothenic acid.

Pantetheine (*Lactobacillus bulgaricus* factor, LBF) has been synthesized previously by Snell, *et al.*,[3] by condensing methyl pantothenate with 2-aminoethyl mercaptan. It also has been shown[4] that LBF can be converted to coenzyme A (CoA) in a liver enzyme system in two hours in about 25% yield. Since the isolation of CoA is tedious, its preparation through enzymatic synthesis seems feasible. Thus a liberal quantity of pantetheine is needed. However, attempts to prepare large amounts of pantetheine by the above method resulted in variable yields of material of inconsistent purity. For this reason a method for the synthesis of β-aletheine[1] was developed. This compound in turn condensed with pantolactone to give LBF in a smooth reaction and with good yields. The reactions carried out are summarized in Fig. 1. The general scheme is somewhat similar to that reported by Baddiley and Thain[5] during the period that the present work was underway. Where comparable, the present work is in agreement with that of the English workers. In addition, the simplified procedure employed herein for preparing carbobenzoxy-β-aletheine, and the device of isolating β-aletheine through its oxalate salt, provide an easy route to this important intermediate.

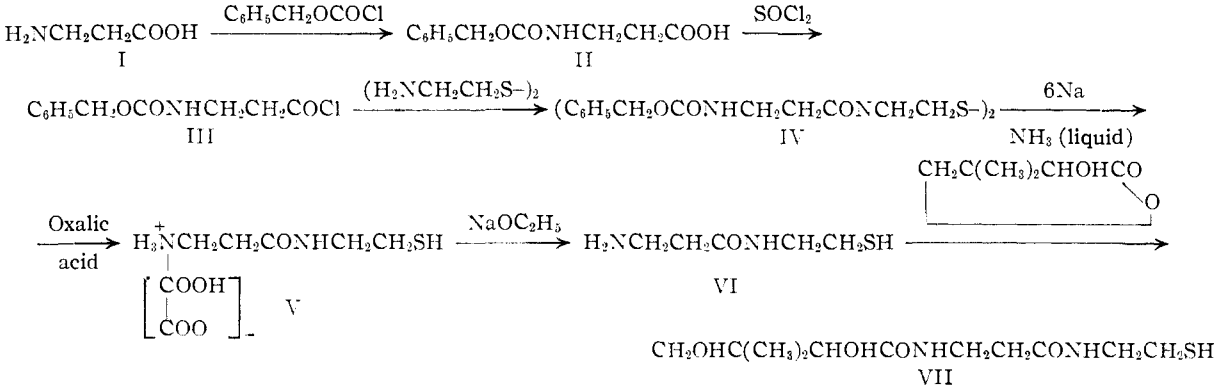


Fig. 1.—Reaction scheme for the synthesis of pantetheine.

Experimental

Carbobenzoxy-β-alanine (II).—This compound was synthesized according to Sifferd and du Vigneaud[6] with the exception that pure β-alanine was the starting material. The product was obtained in 90% yield with a melting point of 103–105° (Sifferd and du Vigneaud reported 102–104°

(1) The name β-aletheine is proposed for N-(β-alanyl)-2-aminoethyl mercaptan and β-alethine for its disulfide form, in conformance with the nomenclature of pantetheine and pantethine, respectively (*cf.* ref. 3).

(2) Paper No. 13 of a series on pantothenic acid studies. Reported in part before The Northwest Regional Meeting of the American Chemical Society, June, 1952. A portion of this work was submitted by C. J. S. for the M.S. degree, June, 1952, Oregon State College. This study has been supported by The Nutrition Foundation, Inc.; The Eli Lilly Laboratories, Inc.; The Abbott Laboratories, Inc.; and the Office of Naval Research, Contract NR 123-058. Published with the approval of the Monographs Publications Committee, Oregon State College, Research paper No. 217, School of Science, Department of Chemistry.

(3) E. E. Snell, G. M. Brown, V. J. Peters, J. A. Craig, E. L. Wittle, J. A. Moore, V. M. McGlohon and O. D. Bird, THIS JOURNAL, **72**, 5349 (1950).

(4) T. E. King and F. M. Strong, *J. Biol. Chem.*, **189**, 325 (1951).

(5) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 800 (1952).

(6) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

before recrystallization). The crude material was sufficiently pure for the preparation of carbobenzoxy-β-alanyl chloride (III). III was made by treatment of II with thionyl chloride[7] and was used immediately in the next step (see following paragraph).

Bis-carbobenzoxy-N-(β-alanyl)-2-aminoethyl Disulfide (IV).—This compound was made by condensation of (III) with 2-aminoethyl mercaptan (prepared according to Mills and Bogert[8]). Two and six-tenths grams of the mercaptan was dissolved in 40 ml. of water and cooled to 0°. Concentrated NaOH solution was added to *p*H 11-12, then the ethereal solution of III from 10 g. of II was added in small portions with stirring. The *p*H was maintained at 11–12 during the reaction. After all the III had been added, stirring was continued for another 15 minutes. The product was separated by filtration, washed with water and crystallized from 95% ethanol; m.p. 153–167°, yield 4.3 g. (45%). After one recrystallization from ethyl acetate, the melting point rose to 180–181° (uncor.) and remained constant upon further recrystallization.

Anal. Calcd. for $C_{26}H_{34}N_4O_6S_2$: C, 55.5; H, 6.09; N, 9.95. Found: C, 56.13; H, 6.26; N, 9.78.[9]

The broad melting range of the product crystallized from ethanol suggested that it might be a mixture of the thiol and the corresponding disulfide forms. An alternative method was thus devised which evidently gave the disulfide form. 2-Aminoethyl mercaptan was dissolved in water and 30% H_2O_2 was added dropwise until a positive nitroprusside test (without cyanide) was no longer given. Then the solution was cooled, made alkaline, and the ethereal solution of III was added as above. The product was separated by filtration and crystallized from ethanol. It melted at 180–182°; yield 80%.

β-Aletheine Oxalate (V).—This was synthesized by reductive cleavage of IV in liquid ammonia by sodium, similar to the method of Sifferd and du Vigneaud[6] for the preparation of sulfur-containing peptides. Three grams of IV was suspended in about 75 ml. of liquid ammonia with stirring. Eight hundred and seventeen mg. of clean sodium was added in small pieces until a slight excess was indicated by a dark blue color. After further stirring for 15 minutes, an equivalent (to the sodium added) amount of ammonium sulfate (2.34 g.) was introduced. The solid material left in the flask after all ammonia evaporated was extracted with boiling absolute alcohol. An alcoholic solution of 1.5 g. of oxalic acid dihydrate was then added to the extract. Crystallization took place in the cold. The needle-like crystals were recrystallized from ethanol. They melted with decomposition at 121–122°; yield 80%.

Anal. Calcd. for $C_5H_{12}N_2OSH_2C_2O_4$: C, 35.29; H, 5.92. Found: C, 34.90, 35.04; H, 6.00, 6.10.[9]

β-Aletheine (VI).—This was prepared by adding an exactly equivalent amount of sodium ethoxide to a methanol solution of the oxalate under nitrogen. After filtering off the sodium oxalate, the solvent was removed *in vacuo*. A white solid remained in the flask with m.p. 93–96° (Baddiley and Thain reported 93–95°[5]). The removal of oxalate by reaction with calcium hydroxide in aqueous solution was not satisfactory, since complete reaction required a rather long time.

Pantetheine (VII).—The formation of pantetheine was accomplished by direct fusion of an intimate mixture of 1.1 mmoles of levorotatory pantolactone and 1.0 mmole of freshly prepared β-aletheine. The mixture melted after a short period of heating at 55–60°. The heating was then maintained for four hours at this temperature. The entire operation was carried out under nitrogen. The unreacted lactone was removed by three successive extractions with small portions of anhydrous ether. The residue was then dissolved in a small amount of methanol and reprecipitated with anhydrous ether. This product, after drying at room temperature under high vacuum, was a colorless, amorphous powder, highly hygroscopic. The average yield in several preparations was approximately 70%.

Anal. Calcd. for $C_{11}H_{22}O_4SN_2$: C, 47.4; H, 7.98; S, 11.5; N, 10.06. Found: C, 47.1; H, 8.17; S, 11.5; N, 9.72, 9.88.[9]

(7) E. Dyer and E. Ballard, THIS JOURNAL, **59**, 1697 (1937).

(8) E. J. Mills and M. T. Bogert, *ibid.*, **62**, 1173 (1940).

(9) Analyzed by Micro-Tech Laboratories, Skokie, Illinois.

The condensation was also carried out in methanol solution, with yields and purity similar to the above.

Microbiological Assays.—Growth of *Lactobacillus fermenti* 9338, *Lactobacillus helveticus* 80, and *Saccharomyces cerevisiae* LM(ATCC No. 9371) were carried out according to previously published methods.[10,11] Bound pantothenic acid assays were performed using *Lactobacillus arabinosus* 17–5[12] after enzymatic digestion of LBF.[13] Assays of pantothenic acid after digestion of LBF samples prepared by the present method have been checked with *L. fermenti* using a pantothenic acid-free medium[10] and the same digestion procedure.[13] This organism has been shown[10] (and confirmed in the present work) to respond only to the free vitamin, either alone or in the presence of LBF.

Results and Discussion

The synthesis of pantetheine by the present method presents certain features that are worthy of special mention. These are: (1) complete utilization of β-aletheine is favored by the use of an excess of pantolactone. The latter compound is sufficiently unlike pantetheine in solubility so that the simple solvent extractions employed are satisfactory for purifying the latter compound. Complete removal of remaining traces of the reactants is generally unnecessary since these have low biological activity in most systems. (2) The steps in the synthesis are simple, straightforward, and few in number. (3) β-Aletheine is easily obtainable in pure form through the oxalate, which crystallizes readily and is easily handled (in contrast to other derivatives such as the hydrochloride, which is extremely hygroscopic in the crude form). Decomposition of the oxalate to free β-aletheine is quantitative. (4) Protection of the thiol group of β-aminoethyl mercaptan is easily accomplished by using it in the disulfide form; subsequent reduction to the thiol form of V is accomplished simultaneously with the cleavage of the carbobenzoxy group of IV.

Attempts to crystallize pantetheine have so far failed. This is not surprising in view of the similar difficulty in crystallizing pantothenic acid. However, purity is indicated not only by analysis but also by biological activity; *L. fermenti* assays indicated that no pantothenic acid had been formed (this organism does not respond to LBF[10]), and bound pantothenic assay showed that the product was at least 95% pure (770 γ pantothenic acid per mg.). Direct assay with *L. helveticus* revealed a potency of over 20,000 LBF units per mg.[14] (range, 18,000 to 28,000, tested at different levels of sample). Values of approximately 20,000 units per mg. have been considered to represent pure material.[15] Finally, the nearly quantitative conversion of pantetheine to S-acetylpantetheine,[16] as measured by both bound pantothenate and acetyl contents, strengthens the belief that the pantetheine prepared herein was essentially pure. (Elementary analyses alone, although correct, may not be a com-

(10) J. A. Craig and E. E. Snell, *J. Bact.*, **61**, 283 (1952).

(11) T. E. King and V. H. Cheldelin, *J. Biol. Chem.*, **174**, 273 (1948).

(12) E. H. Hoag, H. P. Sarett and V. H. Cheldelin, *Ind. Eng. Chem., Anal. Ed.*, **17**, 60 (1945).

(13) J. B. Nielands and F. M. Strong, *Arch. Biochem.*, **19**, 287 (1948).

(14) *L. helveticus* culture and standard yeast extract were kindly supplied by Dr. E. E. Snell.

(15) E. E. Snell, personal communication.

(16) T. E. King, C. J. Stewart and V. H. Cheldelin, *Science*, in press.

pletely valid criterion of purity where the composition of the reaction mixture is similar to that of the product).

For analysis of LBF samples, the bound pantothenic acid assay has proved most satisfactory, since greater precision has been obtainable with this method. Direct analysis of LBF with *L. helveticus*, although giving variable results in our hands, has been used to confirm the identity of the compound since non-LBF materials containing pantothenic acid have occasionally been observed as contaminants in synthetic preparations.[15]

In an autoclaved pantothenic acid free medium,[11] β-aletheine possessed less than 0.5% of the activity of an equivalent amount of β-alanine in supporting growth of yeast. In a filter-sterilized medium, the corresponding activity was less than 0.07%.

β-Aletheine was not inhibitory to the growth of yeast when β-alanine was used as the growth factor. The non-utilization of β-aletheine by yeast is probably due to the impermeability of the cells to this compound. However, an alternative possibility in which pantothenic acid is first formed, and then condensed with the other moieties to give coenzyme A, is not excluded. Evidently, β-aletheine is not identical with the active principle of the incubation mixture of β-alanine with glutamic acid in the presence of resting yeast cells as reported previously.[17]

(17) T. E. King, I. G. Fels and V. H. Cheldelin, *Science*, **71**, 131 (1949).

CORVALLIS, OREGON

[FROM THE CHEMISTRY LABORATORY OF THE STATE UNIVERSITY OF IOWA]

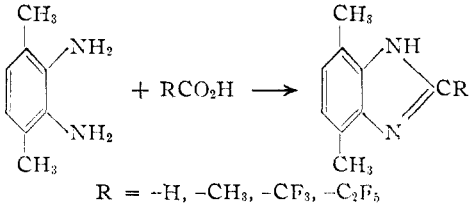
Some Substituted Benzimidazoles

BY WALTER T. SMITH, JR., AND E. C. STEINLE, JR.

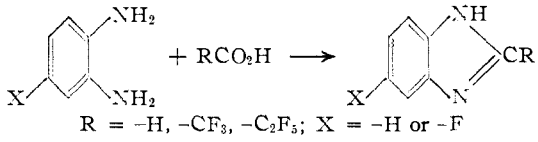
RECEIVED OCTOBER 16, 1952

4,7-Dimethylbenzimidazole, an isomer of the dimethylbenzimidazole isolated from vitamin B_{12}, has been prepared by two methods. In addition, several benzimidazoles of interest have been prepared from the diamines, *o*-phenylenediamine, 3,6-dimethyl-*o*-phenylenediamine and 4-fluoro-*o*-phenylenediamine in condensation with the acids, formic, acetic, fluoroacetic, trifluoroacetic and pentafluoropropionic.

Following the discovery that 5,6-dimethylbenzimidazole was a part of the structure of vitamin B_{12}[1] and that several of the known dimethylbenzimidazoles had varied capacities as growth stimulating agents,[2] it became desirable to prepare the remaining unreported member of the group, 4,7-dimethylbenzimidazole. Since no fluorine-containing benzimidazoles have been reported, it was also desirable to synthesize several representative compounds of this nature. Accordingly, several compounds as represented below have been prepared,



R = -H, $-CH_3$, $-CF_3$, $-C_2F_5$



R = -H, $-CF_3$, $-C_2F_5$; X = -H or -F

Difficulty was experienced in securing a sufficient supply of pure 6-nitro-2,5-xylidine, precursor of the necessary 3,6-dimethyl-*o*-phenylenediamine. It has been reported that 6-nitro-2,5-xylidene melting at 36° can be prepared in 34% yield by the nitration of N-acetyl-2,5-xylidene with mixed acids, followed by saponification of the acetyl group and steam distillation to separate the desired compound.[3] In this investigation, the 34% yield was duplicated, but the product was low-melting and resisted efforts to purify it further and was unsatisfactory for the preparation of stable 3,6-dimethyl-*o*-phenylenediamine. Neither the use of acetyl nitrate or methyl nitrate as nitration agents, nor the substitution of N-formyl-2,5-xylidine as starting material was successful in improving the yield or purity of the product. The following procedure was found to result in a superior product: sulfonation of 2,5-xylidine to give 4-amino-2,5-xylenesulfonic acid, acetylation to 4-acetylamino-2,5-xylenesulfonic acid and nitration of this compound with mixed acids, followed by hydrolysis and steam distillation. By exhaustive steam distillation, a yield of 25% of good quality 6-nitro-2,5-xylidine was isolated and a total yield of 50% was accounted for.

Reduction of 6-nitro-2,5-xylidine with zinc and sodium hydroxide or with sodium hydrosulfite gave the 3,6-dimethyl-*o*-phenylenediamine in sufficient purity (m.p. 72.5–74°) to be reasonably stable, but the yield was fairly low. Consequently, this diamine was usually isolated as the hydrochloride from benzene in 50–60% yield, and used in this form in the preparation of 2-substituted-4,7-dimethylbenzimidazoles.

The dinitration of *p*-xylene is reported to give a mixture of products containing 60–80% of the desired 2,3-dinitro-*p*-xylene.[4] This latter compound would be suitable starting material for the preparation of 4,7-dimethylbenzimidazole, but its separation from 2,6-dinitro-*p*-xylene is impractical. It seemed possible that the separation of the iso-

(1) N. G. Brink and K. Folkers, THIS JOURNAL, **71**, 2951 (1949).

(2) G. Emerson, N. G. Brink, F. W. Holly, F. Koniuszy, D. Heyl and K. Folkers, *ibid.*, **72**, 3084 (1950).

(3) M. H. Wahl, *Ann. chim.*, [11] **5**, 26 (1936).

(4) K. A. Kobe and T. B. Hudson, *Ind. Eng. Chem.*, **42**, 356 (1950).