Anal. Calcd. for $C_{12}H_{17}O_2NS$: C, 60.3; H, 7.1; N, 5.9; S, 13.4. Found: C, 60.1; H, 7.0; N, 5.8; S, 13.2.

Acknowledgments.—We wish to express our appreciation to Dr. H. M. Crooks of Parke, Davis and Co., and to Dr. M. Tishler, of Merck and Co., Inc., who kindly provided generous samples of S-benzyl-DL-penicillamine and of DL-penicillamine, respectively. Our thanks are also due to Mr. R. J. Koegel and his staff for the determination of the elemental analyses.

BETHESDA, MD.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

Synthesis of Ethyl β,β-Diethoxyalanyl-L-cysteinate Hydrochloride[1]

BY HOMER ADKINS[2] AND BURRIS D. TIFFANY[3]

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Ethyl β,β-diethoxyalanyl-L-cysteinate hydrochloride (XIX) has been synthesized through the coupling of N-carbobenzoxy-β,β-diethoxyalanine azide (XI) with S-benzyl-L-cysteine, followed by selective removal of the protecting N-carbobenzoxy and S-benzyl groups by mild reduction. The azide XI was prepared by the carbobenzoxylation of ethyl β,β-diethoxyalanate (IV) followed by conversion of the ester IX to the hydrazide X and then to the azide. The primary coupling product, N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine (XIV) and (XV), was separated into its component D,L- and L,L-diastereoisomers by fractional crystallization of its methyl esters and sodium salts. D,L-N-Carbobenzoxy-β,β-diethoxyalanylglycine (XII) resulted from a preliminary investigation of the synthetic route using glycine. An attempt to couple the unprotected β,β-diethoxyalanine azide (VI) with glycine yielded 3-diethoxymethyl-2,5-piperazinedione (VIII).

In connection with a study of the structure of benzylpenicillin,[4] it was of interest to synthesize the stereoisomeric ester hydrochlorides (I) of β,β-diethoxyalanylpenicillamine, a dipeptide incorporating certain structural features known to be present in benzylpenicillin itself.

$(CH_3CH_2O)_2CHCHCONHCHCO_2CH_2CH_3$ I, R = CH_3
$H_2N\cdot HCl$ CR_2SH II, R = H

This paper describes the synthesis of one of the stereoisomers of the lower homolog, ethyl β,β-diethoxyalanyl-L-cysteinate hydrochloride (II), derived from L-cysteine, as a model for the synthesis of the dipeptide ester hydrochlorides I, which require the less available penicillamine.

The first approach studied was the azide coupling of β,β-diethoxyalanine (III) with ethyl glycinate. The latter amino acid was employed in developing the method to avoid difficulties due to the sensitive sulfhydryl group and the complications of diastereoisomeric dipeptides.

Ethyl β,β-diethoxyalanate (IV), prepared from ethyl glycinate,[5] was converted to the hydrazide V in good yield, using anhydrous hydrazine. The hydrazide was surprisingly stable, considering the functional groups present, and could be distilled at reduced pressure without change.

We were encouraged by the unusual stability of the hydrazide to attempt direct conversion to the azide VI and coupling with glycine without protecting the amino group. When this unconventional series of reactions was tried, the product, obtained in 10% yield, was not the dipeptide ester VII, however, but a white water-soluble solid which gave correct carbon and hydrogen values for 3-diethoxymethyl-2,5-piperazinedione (VIII) *i.e.*, the compound to be expected upon cyclization of the coupled product VII. The failure, therefore, appeared not to be in the coupling, although of low yield, but rather in preventing the subsequent cyclization. Herein is implied the existence of a moderately stable aliphatic primary amino acid azide, which seems to be unprecedented.[6]

For a successful coupling procedure we turned to Bergmann's method for protection of the amino group. Carbobenzoxylation of ethyl β,β-diethoxyalanate (IV) was accomplished in 81% yield, and the resulting ethyl N-carbobenzoxy-β,β-diethoxyalanate (IX) was converted readily to the hydrazide X with 85% hydrazine hydrate in ethanol. Before treatment with nitrous acid this acetal-containing hydrazide was tested for stability in aqueous acid. It could be dissolved in three equivalents of cold 1 *N* hydrochloric acid and largely recovered unchanged upon neutralization of the acid. The azide XI was prepared from the hydrazide using nitrous acid and coupled with glycine in potassium carbonate solution, giving N-carbobenzoxy-β,β-diethoxyalanylglycine (XII) as a white crystalline solid in good yield. The crystalline methyl ester XIII was prepared readily from the acid upon treatment with diazomethane.

Having demonstrated that the acetal system would survive in the coupling reaction, attention was next turned to the dipeptide containing the L-cysteine residue. The sensitive sulfhydryl group was protected with a benzyl group as described by Wood and du Vigneaud.[7] When the coupling procedure was carried out with S-benzyl-L-cysteine, the product, obtained in 80% yield, was a solid with a broad melting range and presumably was a mixture of the D,L- and L,L-diastereoisomers of the dipeptide XIV and XV.

For separation of the diastereoisomers the mixture of acids was converted to the methyl esters

(1) From a thesis submitted by Burris D. Tiffany to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1949.

(2) Deceased August 10, 1949.

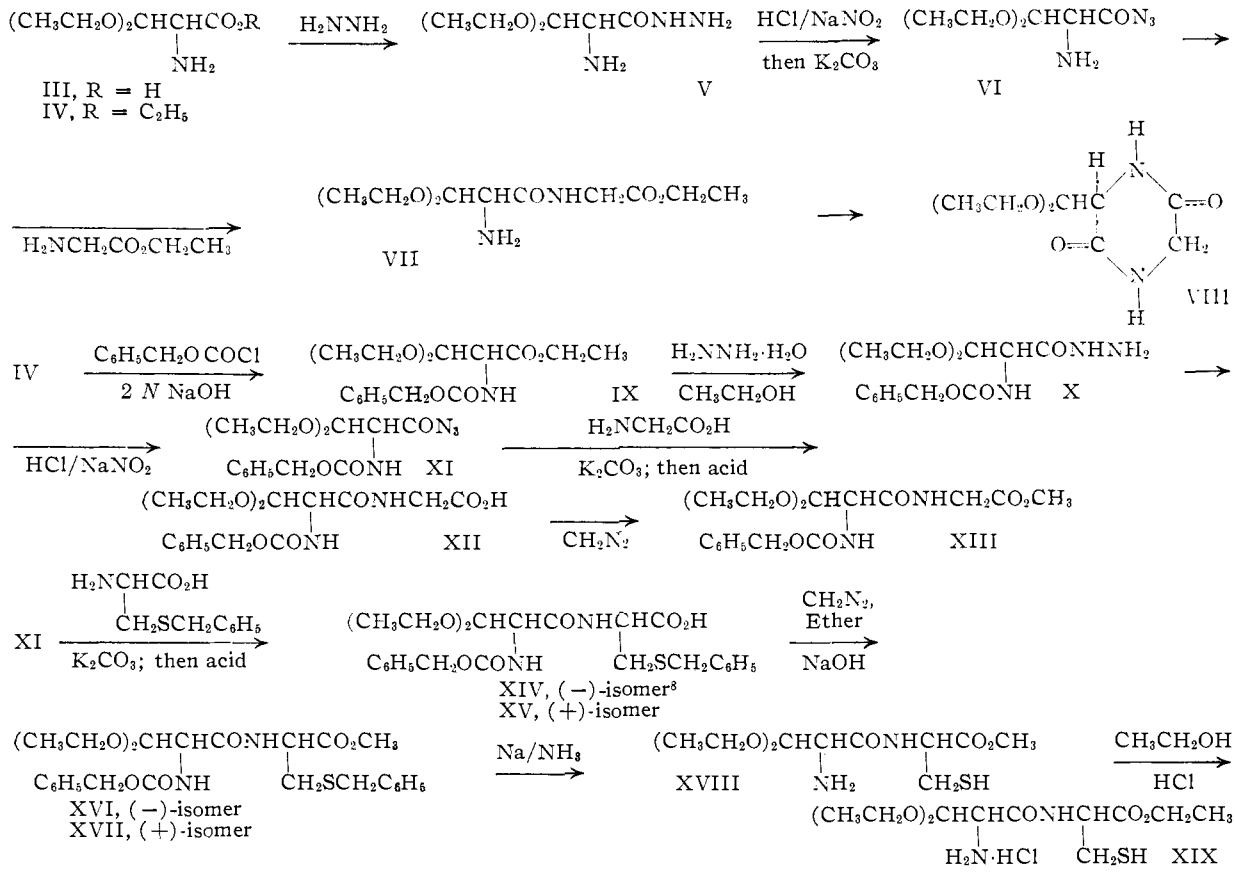
(3) The Upjohn Co., Kalamazoo, Mich. Wisconsin Alumni Research Foundation Research Assistant, 1947–1949.

(4) *Cf.* H. Adkins, R. M. Ross and D. C. Schroeder, THIS JOURNAL, **72**, 5401 (1950).

(5) "The Chemistry of Penicillin," Clarke, Johnson and Robinson, Editors, Princeton University Press, Princeton, N. J., 1949, p. 312.

(6) *Cf.* P. A. S. Smith's review of the Curtius reaction in "Organic Reactions," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1946, p. 353.

(7) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).



XVI and XVII with diazomethane, and recrystallized from a mixture of benzene and petroleum ether in which the dextrorotatory ester was the less soluble. Material of good quality was obtained in 25% over-all yield from the hydrazide (based on the total amount of all isomers expected). Hydrolysis of the ester to the free acid apparently was accompanied by some decomposition or partial racemization, since material of sharp melting point could not be obtained. Attempts to separate the isomers by fractional recrystallization of the acids instead of the esters was not promising.

The levorotatory form of the dipeptide XIV was obtained by hydrolysis of the residual methyl esters from which the dextrorotatory ester had been crystallized, extraction of the *sodium salts* into chloroform and fractional recrystallization of these salts from absolute ethanol. The alkali salts of these isomeric acids showed a remarkable solubility in chloroform, being much more soluble in this solvent than in water. Furthermore, the sodium salt of the levorotatory form, which was isolated in a fairly pure state, had a surprisingly low melting point, 169–173°. The levorotatory free acid XIV was prepared from a dilute aqueous solution of the salt by treatment with dilute acid and was recrystallized from chloroform. Reaction with diazomethane converted it to the crystalline methyl ester (XVI).

Both protecting groups in the dipeptides, the N-carbobenzoxy and the S-benzyl groups, were removed simultaneously upon reduction with sodium in liquid ammonia. The process was successful however, only when applied to the methyl ester and not with the free acid as suggested by the literature.[9] Hopkins mercuric sulfate reagent, which usually is successful for separation of cysteine-containing peptides, failed to precipitate the product in either case here. The product from reduction of the dextro methyl ester was a crystalline solid but apparently a mixture. By means of an ester interchange in ethanolic hydrogen chloride, however, it was converted to a homogeneous product consisting of the ethyl ester hydrochloride, ethyl β,β-diethoxyalanyl-L-cysteinate hydrochloride (XIX), a sensitive white crystalline solid. A test with sodium nitroprusside reagent verified the presence of the sulfhydryl group and a Zeisel determination indicated the presence of three ethoxyl groups. The free amino group was evidenced by the formation of a hydrochloride.

Acknowledgment.—We wish to express our appreciation to Dr. A. L. Wilds for advice concerning the preparation of the manuscript.

Experimental[10]

β,β-Diethoxyalanine Hydrazide (V).—Ethyl β,β-diethoxyalanate (IV) required in this preparation was prepared from sodium ethyl α-N-formylamino-β-hydroxyacrylate in 30% yield by a procedure described in a report from Charles

(8) In these compounds (+) and (−) are used to designate an observed rotation with no implication regarding actual configurations. Small capital D and L are employed as usual to designate known configurations.

(9) C. R. Harington and R. V. P. Rivers, *Biochem. J.*, **38**, 417 (1944).

(10) All melting points are corrected. Microanalyses were carried out by Richard Hunt and Edward Shiner and by the Clark Microanalytical Laboratory, Urbana, Ill.

Pfizer and Co., Inc.[5]; however, the results were not readily reproducible. The sodium enolate was prepared from ethyl N-formylglycinate in 97% yield (crude) as described in a report from the Northern Regional Research Laboratories.[6]

In a stoppered 25-ml. erlenmeyer flask, a mixture of 10.25 g. (0.050 mole) of ethyl β,β-diethoxyalanate and 2.4 g. (0.075 mole) of anhydrous hydrazine[11] was kept under nitrogen at room temperature with occasional shaking for one day, whereupon it crystallized to a dense mass when seeded. Upon treatment with Norit and recrystallization twice from ether, this produced 7.55 g. (79%) of white needles, m.p. 63–65°. The product could be distilled at 111–112° (0.2 mm.), giving a solid which, upon further recrystallization from ether, melted at 64.5–65.5° and showed no m.p. depression with the solid before distillation.

Anal. Calcd. for $C_7H_{17}N_3O_3$: C, 43.97; H, 8.96; neut. equiv., 191. Found: C, 44.09; H, 9.16; neut. equiv., 190, 193.

3-Diethoxymethyl-2,5-piperazinedione (VIII).—A solution of 0.38 g. (0.0020 mole) of β,β-diethoxyalanine hydrazide in 5 ml. of water was treated, while swirling in an ice-salt-bath, with 10 drops (0.004 mole) of concentrated hydrochloric acid followed immediately by 0.14 g. (0.0020 mole) of sodium nitrite in 3 ml. of water. The solution was treated with 0.42 g. (0.0030 mole) of potassium carbonate, the azide quickly extracted well into ethyl acetate (Mallinckrodt analytical reagent), dried over anhydrous sodium sulfate and added to a dried ethereal extract of ethyl glycinate, previously prepared from 0.28 g. (0.0020 mole) of ethyl glycinate hydrochloride and 0.28 g. (0.0020 mole) of potassium carbonate. After one day at room temperature the resulting solution was concentrated under reduced pressure to give several milligrams of white crystalline solid in a small amount of pale yellow oil. Stirring with 50 ml. of ether removed the oil, leaving 0.045 g. (10%) of water-soluble solid, m.p. 176–177.5° without recrystallization.

Anal. Calcd. for $C_9H_{16}N_2O_4$: C, 50.00; H, 7.40. Found: C, 49.76; H, 7.29.

Ethyl N-Carbobenzoxy-β,β-diethoxyalanate (IX).—The preparation of this compound by procedures differing from the one given below has been described previously.[12] In a 250-ml. three-necked flask equipped with a mechanical stirrer and two dropping funnels was placed 23.3 g. (0.100 mole) of ethyl β,β-diethoxyalanate, rinsing in with 23 ml. of water. This was stirred and cooled in an ice–salt-bath while 20.0 g. (0.115 mole) of carbobenzoxy chloride[13] and 60 ml. of 2 *N* sodium hydroxide were added simultaneously during 25 minutes causing an orange oil to separate. After an additional 15 minutes of cooling and stirring, the mixture was extracted with three 100-ml. portions of ether and these washed with water, dried over sodium sulfate, filtered and concentrated under reduced pressure. Distillation of the resulting 29.4 g. of brown oil produced 27.4 g. (81%) of a pale yellow viscous oil, b.p. 164–168° (0.2 mm.), $n^{25}D$ 1.4903.

N-Carbobenzoxy-β,β-diethoxyalanine Hydrazide (X).—In a 125-ml. erlenmeyer flask 27.35 g. (0.080 mole) of ethyl N-carbobenzoxy-β,β-diethoxyalanate, 12 ml. of 85% hydrazine hydrate and 25 ml. of absolute ethanol were shaken until homogeneous and kept at room temperature for 20 hr. The crystals which formed were collected, rinsed with 20 ml. of 50–50 ethanol–ether and dried, giving 16.5 g. of solid, m.p. 139.5–140.5°. The filtrate yielded an additional 6.0 g., m.p. 139.5–140.5°, and 0.6 g., m.p. 134–136°, for a total of 23.1 g. (89%). A sample was recrystallized from ethanol–ether for analysis, m.p. 139.5–140.5°.

Anal. Calcd. for $C_{15}H_{23}N_3O_5$: C, 55.33; H, 7.13. Found: C, 55.47; H, 7.03.

Stability of N-Carbobenzoxy-β,β-diethoxyalanine Hydrazide (X) toward 1 *N* Hydrochloric Acid.—In 3 ml. of 1 *N* hydrochloric acid[14] at 0–5° was dissolved 0.34 g. (0.0010 mole) of N-carbobenzoxy-β,β-diethoxyalanine hydrazide, m.p. 139.5–140.5°. After 5 minutes at this temperature the solution was made alkaline by slow addition of a solution of 0.28 g. (0.0020 mole) of potassium carbonate in 3 ml. of water. The white flocculent precipitate which separated was collected on a filter, dried and recrystallized from absolute ethanol. A total of 0.25 g. of solid, m.p. 139.5° and mixed m.p. with starting material, 138–139°, was obtained.

N-Carbobenzoxy-β,β-diethoxyalanylglycine (XII).—A mixture of 1.00 g. (0.0030 mole) of N-carbobenzoxy-β,β-diethoxyalanine hydrazide and 9 ml. (0.0090 mole) of 1 *N* hydrochloric acid was stirred mechanically at 0–5° for 3 minutes until the hydrazide was dissolved and then 10 ml. of dioxane added. Immediately a solution of 0.25 g. (0.0036 mole) of sodium nitrite in 3 ml. of water was added with stirring over a period of one minute, followed quickly by the addition of a solution of 0.45 g. (0.0060 mole) of glycine and 1.38 g. (0.010 mole) of potassium carbonate in 5 ml. of water. Stirring was continued 1 hr., the latter half without cooling. The resulting clear solution was concentrated under reduced pressure at less than 45°. The gelatinous residue was added to 15 ml. of water and the resulting suspension filtered and dried, giving 0.05 g. of solid, m.p. 142–148°. Extraction of the clear filtrate with ether yielded 0.015 g. of additional solid, m.p. 149–150°. Upon acidification of the aqueous layer, extraction of the oil which separated with ether and concentration of the extracts, there was obtained 1.09 g. of tiny white needles, m.p. 111–115°. This was dissolved in 3 ml. of ethanol, diluted with 20 ml. of hot water and seeded. Upon cooling, the solution became milky and tiny needles replaced this milkiness only on long standing at room temperature. The needles, when collected, weighed 0.96 g. (83%), m.p. 119–120°. Further recrystallization raised the m.p. to 119.5–121°.

Anal. Calcd. for $C_{17}H_{24}N_2O_7$: C, 55.43; H, 6.57; neut. equiv., 368. Found: C, 55.59; H, 6.44; neut. equiv., 378.

Methyl N-Carbobenzoxy-β,β-diethoxyalanylglycinate (XIII).—A suspension of 0.37 g. (0.0010 mole) of N-carbobenzoxy-β,β-diethoxyalanylglycine, m.p. 120–122°, in 50 ml. of ether was treated in portions with excess ethereal diazomethane[15] causing gas evolution as the suspended solid dissolved. After 20 minutes at room temperature the solution was concentrated to 2 ml., and the rosettes which then slowly formed were collected and dried, giving 0.35 g. (92%) of white crystals, m.p. 94–96°. A sample was recrystallized from ether–petroleum ether (40–60°), m.p. 94.5–96°.

Anal. Calcd. for $C_{17}H_{24}N_2O_7$: C, 56.54; H, 6.85. Found: C, 56.83; H, 7.09.

N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine (XIV and XV).—A mixture of 26.1 g. (0.080 mole) of N-carbobenzoxy-β,β-diethoxyalanine hydrazide, 240 ml. (0.240 mole) of 1 *N* hydrochloric acid and 240 ml. of dioxane was stirred mechanically at 0°, whereupon the hydrazide dissolved rapidly. After 10 minutes a solution of 6.6 g. (0.096 mole) of sodium nitrite in 72 ml. of water was added with continued stirring at 0° during one minute causing a cloudiness. Immediately, a previously prepared solution of 21.1 g. (0.100 mole) of S-benzyl-L-cysteine[7] and 41.5 g. (0.300 mole) of potassium carbonate in 120 ml. of water was added over about 4 minutes at 0–5°. The resulting oily suspension was stirred with cooling for 30 minutes and without cooling for another 60 minutes, whereupon it became an essentially clear yellow solution which was then concentrated under reduced pressure to a thick white paste of tiny needles. These were suspended in 700 ml. of water and stirred with 150 ml. of chloroform into which they were quickly extracted.[16] Without separating the layers, the mixture was acidified to congo red with about 160 ml. of 3 *N* hydrochloric acid and the extraction of the resulting free organic acid completed with two additional 120-ml. portions of chloroform. The combined extract was washed with two 100-ml. portions of water, dried over sodium sulfate, filtered and concentrated under reduced pressure on the steam-bath. The clear red residue (40 g.) crystallized from a mixture of 75 ml. of benzene and 110 ml. of petroleum ether (60–68°) only as a gelatinous precipitate which was

(11) L. I. Smith and K. L. Howard, *Organic Syntheses*, **24**, 53 (1944).

(12) Reference 5, p. 518.

(13) H. E. Carter, R. L. Frank and H. W. Johnson, *Org. Syntheses*, **23**, 13 (1943).

(14) It was found that in all cases three equivalents of strong acid was required to effect complete solution of this hydrazide.

(15) F. Arndt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., p. 166, note 3.

(16) It should be noted here that the sodium and potassium salts of the product were more soluble in chloroform than in water even though this would not normally be expected.

used directly in the preparation of the methyl ester described below.

In a similar experiment starting with 11.3 g. (0.035 mole) of the hydrazide there was isolated 14.1 g. (80%) of a solid, m.p. 95–109°, which was presumably a mixture of diastereoisomers.

Anal. Calcd. for $C_{25}H_{32}N_2O_7S$: neut. equiv., 504. Found: neut. equiv., 513.

In another experiment starting with 32.5 g. (0.100 mole) of the hydrazide, only 15.9 g. (31%) of crystalline solid was obtained, but this was apparently largely the (+)-isomer since it was subsequently converted to the (+)-methyl ester, m.p. 131–132°, $[\alpha]^{27}D$ 17.8° (*c* 2.8, chloroform) in 82% yield.

(+)-Methyl N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate[3] **(XVII).**—A solution of the crude N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine from the preceding preparation in 150 ml. of benzene was treated slowly with excess diazomethane in ether while stirring at 5°, causing the separation of a crystalline precipitate. This was collected after 20 hr. at room temperature, washed with a mixture of benzene and petroleum ether (40–60°) and dried. The 24 g. of crystals, m.p. 116–120° with softening at 110°, was recrystallized from a mixture of benzene and petroleum ether (90–100°) to afford 10.1 g. (25% calculated from the hydrazide and based on the total amount of all isomers possible) of white crystals, m.p. 130–131.5°. Two similar runs gave yields of 26%, m.p. 131–132°, and 27%, m.p. 130–131°. Further recrystallization raised the m.p. to 131.0–131.5°, $[\alpha]^{27}D$ +17.8°, +17.9° (*c* 2.8, 1.8, chloroform).

Anal. Calcd. for $C_{26}H_{34}N_2O_7S$: C, 60.23; H, 6.61. Found: C, 60.25; H, 6.44.

The filtrates from above were concentrated to yield 11.7 g. of crystals, m.p. 109–112°, $[\alpha]^{27}D$ −9.7°, and 1.8 g. of amorphous solid which were used without further purification in the preparation of (−)-sodium N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate described below.

(−)-Sodium N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate.—The 11.7 g. of solid, m.p. 109–112°, and 1.8 g. of amorphous methyl N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate from the preceding preparation were dissolved in 40 ml. of dioxane with slight warming, cooled to room temperature and treated with 8 ml. of 5 *N* sodium hydroxide. After 50 minutes with occasional shaking at room temperature, the two-phase system was diluted with 400 ml. of water, causing first a clear solution and then an oily suspension. This was extracted well with chloroform (in which sodium N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate is quite soluble) and the combined extract washed with two 20-ml. portions of water, dried over sodium sulfate, filtered and concentrated to a viscous yellow sirup. The latter crystallized from 40 ml. of absolute ethanol at room temperature upon seeding and then diluting with 30 ml. of petroleum ether (60–68°) when rosettes began to grow. After two days the crystals were collected, rinsed with a mixture of ethanol and petroleum ether (40–60°) and dried, affording 2.0 g., m.p. 169–173°, $[\alpha]^{25}D$ −18.6° (*c* 1.7, chloroform). From the concentrated filtrate was obtained 1.7 g. of fluffy needles, m.p. 167–171°, and from the original aqueous layer an additional 1.5 g., m.p. 165–170°.

(−)-N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine (XIV).—Several samples of (−)-sodium N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate representing material melting in the range of 165–175° were combined (2.7 g., 0.0051 mole) and dissolved in 75 ml. of water by warming. The gelatinous precipitate which resisted solution was removed by filtration and the clear solution acidified by dropwise addition of 6 *N* hydrochloric acid producing an oily suspension which coagulated and solidified overnight. The solid was collected, pressed on the filter, dissolved in chloroform, dried over sodium sulfate and concentrated to an almost colorless sirup which changed to a suspension of fine white crystals when ether was added. Upon isolating a small portion (155 mg.) of these crystals, triturating them with ether, collecting and drying, they melted at 126.0–126.5°, $[\alpha]^{29}D$ −13.5° (*c* 1.6, chloroform).

Anal. Calcd. for $C_{25}H_{32}N_2O_7S$: C, 59.53; H, 6.38. Found: C, 59.46; H, 6.51.

(−)-Methyl N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate (XVI).—The bulk of the ether suspension of (−)-N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine from the preceding preparation (0.0048 mole) was treated in portions with excess ethereal diazomethane while cooling and swirling, resulting in gas evolution but no visible change in the suspended material. After standing overnight the suspension was concentrated, petroleum ether (40–60°) added, the suspension concentrated again to 40 ml. and the fine white precipitate collected and dried. The 2.1 g. (84%) of solid melted at 114.5–116° with softening at 112°, $[\alpha]^{27}D$ −16.9° (*c* 2.1, chloroform). An additional 0.10 g. (88% total), m.p. 111–114°, was obtained from the filtrate. A sample which was recrystallized by dissolving in ether and adding petroleum ether (40–60°) melted at 116.0–116.5°.

Anal. Calcd. for $C_{26}H_{34}N_2O_7S$: C, 60.23; H, 6.61. Found: C, 60.49; H, 6.65.

(+)-N-Carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine (XV).—In a 25-ml. erlenmeyer flask 0.52 g. (0.0010 mole) of (+)-methyl N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate, m.p. 131–132°, $[\alpha]^{27}D$ 17.8° (*c* 2.8, chloroform), was dissolved with stirring and slight warming in 3 ml. of dioxane. Then 1.5 ml. (0.0015 mole) of 1 *N* sodium hydroxide was added dropwise with stirring over a period of 5 minutes and stirring continued 45 minutes longer, the solution becoming essentially clear after 15 minutes. Gradual dilution with 20 ml. of water produced a cloudiness which was removed by treating with Norit and filtering. The clear solution was acidified to congo red by dropwise addition of 1 *N* hydrochloric acid with rapid stirring and the white gelatinous precipitate which separated was stirred 45 minutes, filtered, washed with water and sucked dry on the filter affording 0.45 g. (89%), m.p. 118–122°. Another run in which the precipitated acid was extracted into chloroform, dried, concentrated and recrystallized from a mixture of benzene and petroleum ether (60–68°) yielded 0.29 g. (57%), m.p. 129–133°, which could not be improved by further recrystallization.

(+)-Ethyl β,β-Diethoxyalanyl-L-cysteinate Hydrochloride (XIX).—In a 250-ml. three-necked flask equipped with a mechanical stirrer, drying tube containing soda-lime and nitrogen inlet was placed 40 ml. of liquid ammonia and 1.04 g. (0.0020 mole) of (+)-methyl N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteinate, m.p. 130–131.5°, and a pinch of iron rust added. While the suspension was stirred, 0.18 g. (0.008 mole) of sodium was added in 0.02-g. pieces during two minutes, the characteristic blue color disappearing almost immediately after each addition until the reduction was complete, whereupon the color persisted. (During this process the original white suspension first dissolved completely and then a new white suspension formed.) Almost immediately, 0.70 g. (0.005 mole) of ammonium sulfate was added, whereupon the color gradually disappeared. The ammonia was evaporated under a stream of nitrogen during 1 hr., the white residue treated with 2 ml. of water and the gray solution neutralized to Alkacid test paper with 42 drops of 6 *N* sulfuric acid which produced a short-lived gas evolution and a strong odor of hydrogen sulfide at each addition. While the neutral solution was stirred, it was treated slowly with 25 ml. of absolute ethanol, causing the separation of a white granular precipitate which was removed by filtration and discarded. The clear colorless filtrate was concentrated under reduced pressure below 40° to a yellow sirup (which always crystallized spontaneously in later runs). It was dissolved in 30 ml. of absolute ethanol, this saturated with anhydrous hydrogen chloride while cooling and after 2 hr. at room temperature concentrated under reduced pressure. Fresh solvent was added and this also removed, leaving an orange moist cake of crystals which was triturated with 1.5 ml. of methyl ethyl ketone. The solid was collected on a filter stick, rinsed with 1 ml. of methyl ethyl ketone, then with ether and dried under diminished pressure at 70°. The almost white crystalline product weighed 249 mg., m.p. 168–170° (micro block), $[\alpha]^{29}D$ +25.5° (*c* 0.64, chloroform).

Anal. Calcd. for $C_{12}H_{25}N_2O_5SCl$: C, 41.80; H, 7.28; alkoxyl (calcd. as 3 ethoxyl), 39.25. Found: C, 42.17; H, 7.01; alkoxyl (ethoxyl), 39.58.

A sample of the product dissolved in 10% sodium hydroxide and treated with aqueous sodium nitroprusside

solution gave a deep red color indicating the presence of a sulfhydryl group.

Hopkins reagent was of no value in isolating this product. When added to an acidified aqueous solution of the residue after the liquid ammonia was removed, it sometimes caused a yellow color but gave no precipitate. The final crystalline hydrochloride also failed to give a precipitate with this reagent.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, U. S. PUBLIC HEALTH SERVICE, DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Studies on the Occurrence and Structure of Acetylandromedol (Andromedotoxin)

BY W. H. TALLENT, MARY L. RIETHOF AND E. C. HORNING

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A procedure for the detection of acetylandromedol in plant extracts was developed through use of paper electrophoresis with borate buffer solutions. Positive results were found for some but not all species of *Kalmia, Leucothoe, Lyonia, Pernettya, Pieris* and *Rhododendron*. A particularly good source was found in *K. angustifolia* var. *caroliniana*. New evidence indicated that the empirical formula for andromedol is $C_{20}H_{34}O_6$, and that acetylandromedol, grayanotoxin I and rhodotoxin are identical and have the formula $C_{22}H_{36}O_7$.

The reported toxicity to stock of *Andromeda, Kalmia, Leucothoe* and *Lyonia* species indicates that a number of plants of the *Ericaceae* in addition to those of the genus *Rhododendron* contain physiologically active compounds. Acetylandromedol,[1] isolated[2] from leaves of *Rhododendron maximum*, was found to be a potent hypotensive agent,[3] and in order to determine if commonly occurring species of these genera contained acetylandromedol or related substances a survey was made of a number of plants of the *Ericaceae* with reported or potential physiological activity. These included *Andromeda japonica* from which Eykman[4] isolated in 1882 a crude toxic substance to which the name asebotoxin was given, and *Rhododendron luteum*, reported by Xenophon to be the source of a poisonous honey given to Grecian soldiers in an early campaign in Asia Minor. It was necessary to develop a method for the identification of acetylandromedol in plant samples, and this work is described in Part I together with the results of a limited survey made for ten genera in the *Ericaceae*.

Part II describes further chemical studies on acetylandromedol.

I. Plant Studies

Acetylandromedol is known to contain a 1,2-glycol structure.[2] This suggested the possibility of utilizing paper electrophoresis with a borate electrolyte for the separation and identification of plant components in the acetylandromedol fraction. The rate of migration of a borate diol complex during paper electrophoresis depends on the borate-diol equilibrium and upon the absorption characteristics of the complex with regard to cellulose. In dilute borate solutions a 2:1 diol–borate complex may be formed in addition to a 1:1 complex, and this effect usually can be recognized by studying the effect of borate concentration changes. The 2:1 diol–borate complex is the stronger acid of the two.[5]

It was found that acetylandromedol migrated toward the anode on paper electrophoresis with sodium tetraborate solutions. The vanillin–perchloric acid spray of Godin[6] was an excellent visualizing reagent. With 0.05 *M* sodium tetraborate concentration, a single migrating species was observed (curve B, Fig. 1), and with 0.01 *M* electrolyte concentration two ionic species were observed (1a and 1b in curve D, Fig. 1). The principal species under the latter conditions was assumed to be the expected 1:1 borate-diol complex. The secondary species with higher mobility occurring in very dilute borate solution was assumed to be the 2:1 complex. An alternate but less likely explanation for the two ionic species is that they represent borate complexes with two different pairs of hydroxyl groups of acetylandromedol.[5a,5e]

When this procedure was used with crude acetylandromedol fractions from *Rhododendron maximum* leaves, the characteristic behavior of acetylandromedol with different borate concentrations was exhibited (curves A and C in Fig. 1), and compounds responsible for two additional colored areas (marked 2 and 3 in curves A and C) were indicated by the spray reagent. The recognition of these and other compounds in successive runs was aided materially by the strong and distinctive colors produced by the Godin reagent.

This procedure was applied to a number of plant specimens from the U. S., Cuba and Costa Rica. Figure 2 shows examples of the results obtained after electrophoresis. Data taken from such electrophoretograms are summarized in Table I. The presence of acetylandromedol was checked, where it occurred, in two concentrations of sodium tetraborate electrolyte (0.01 and 0.05 *M*) in order to confirm the characteristic equilibrium behavior.

(1) This is proposed in Part II as a change in name from andromedotoxin.

(2) H. B. Wood, V. L. Stromberg, J. C. Keresztesy and E. C. Horning, THIS JOURNAL, **76**, 5689 (1954).

(3) N. C. Moran, P. E. Dresel, M. E. Perkins and A. P. Richardson, *J. Pharmacol. Exp. Therap.*, **110**, 415 (1954); N. C. Moran, M. E. Perkins and A. P. Richardson, *ibid.*, **111**, 454 (1954). In normal dogs, intravenous administration of 5–10 mcg./kg. led to 20–40% lowering of blood pressure. Levels required for hypotensive action in humans were slightly higher.

(4) J. F. Eykman, *Rec. trav. chim.*, **1**, 225 (1882).

(5) Details on the effect of borate concentration and other factors on borate-diol complex formation are given in (a) H. S. Isbell, J. F. Brewster, N. B. Holt and H. L. Frush, *J. Research Nat. Bur. Standards*, **40**, 129 (1948); (b) J. Boeseken, *Advances in Carbohydrate Chem.*, **4**, 189 (1949); (c) C. A. Zittle, *Advances in Enzymology*, **12**, 493 (1951); R. Consden and W. M. Stanier, *Nature*, **169**, 783 (1952), and (e) D. J. Bell and D. H. Northcote, *Chemistry & Industry*, 1328 (1954).

(6) P. Godin, *Nature*, **174**, 134 (1954).