Synthesis of [4′,4′-Bis(glycine),5′,5′-bis(valine)]actinomycin D, a Tetra-*N*-demethylactinomycin[1a,b]

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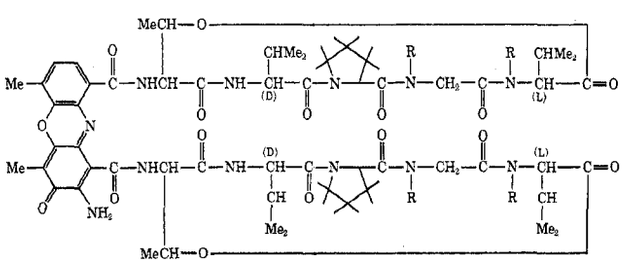
An analog of actinomycin D, lacking the four *N*-methyl groups in the peptide lactone rings, has been synthesized by a route involving *first* the cyclization of the tetra-*N*-demethyl linear pentapeptide; condensation of this cyclic peptide with the benzoyl moiety, followed by successive reduction and oxidation by standard procedures, led to [Gly⁴′,Val⁵′]actinomycin D. CD spectra indicate that this analog has a conformation different from that of actinomycin D in methanol, chloroform, and acetonitrile solutions, but a very similar conformation in hexafluoroacetone. Biologically, [Gly⁴′,Val⁵′]actinomycin D showed no antimicrobial or cytotoxic activity in several tests.

Because several members of the actinomycin group, particularly actinomycins C_3 and D (C_1),[1c] have shown considerable activity as antitumor agents, studies to elucidate their structures, to characterize them completely, and to synthesize them and numerous analogs have been reported, notably by Brockmann and coworkers[2] and Meienhofer.[3] We are now reporting our synthesis of a tetra-*N*-demethyl analog, [4′,4′-bis(glycine),5′,5′-bis(valine)]actinomycin D (**1a**), in which



1a, R = H, [Gly⁴′, Val⁵′]actinomycin D
b, R = CH_3, actinomycin D

the four *N*-methyl groups of the cyclic peptide lactones in actinomycin D have been replaced by hydrogens.

Heretofore, reported syntheses involved condensation of linear peptide with a benzoyl moiety, followed by cyclization of the peptide chain, either *via* ester bond formation (lactonization)[2c,2e,2f] or *via* peptide bond formation.[2a,2b,2d,3b] Cyclization of the peptide by either method was either preceded or followed by formation of the chromophore involving catalytic hydrogenation and finally controlled oxidation. We proposed a slightly different approach, *i.e.*, *first* cyclizing a suitably protected linear peptide **9**, *via* peptide bond formation, and then condensing the deblocked cyclic peptide **14** with the benzoyl residue, followed finally by hydrogenation and oxidation[4] (Scheme I).

The first steps were suggested by Meienhofer's synthesis[3b,5] but differed in methods of coupling.

The benzyl ester of *tert*-butyloxycarbonyl-L-threonine (**2**) was prepared by the method of Baer.[6] After removal of the major portion of excess benzyl chloride, dry pyridine was added and the mixture was heated to quaternize the last traces of benzyl chloride. Condensation of **2** with benzyloxycarbonyl-L-valine by the DCC method, in acetone containing dry pyridine, afforded the protected didepsipeptide (**3a**) as a colorless syrup, which was hydrogenolyzed to yield the partially deblocked **3b**. Crystallization from water gave the purified material, with overall yields of 55–70%, based on **2**. Action of the active ester, benzyloxycarbonylglycine *N*-hydroxysuccinimide,[7] on the triethylamine salt of **3b** gave **4**, a sticky material, which was used directly in the next step. A small amount was converted to the crystalline, analytically pure dicyclohexylammonium salt. A salt of similar purity was prepared from the product obtained by DCC coupling of benzyloxycarbonylglycine with **3b**. Hydrogenolysis of **4** produced **5**, a crystalline solid, which was converted to **6**, also an analytically pure solid, *via* condensation with benzyloxycarbonyl-L-proline-*N*-hydroxysuccinimide ester. Hydrogenolysis of **6** gave the partially blocked tetrapeptide **7**. Since this tetrapeptide swelled in organic solvents to a gelatinous material, it was not purified but was used as the air-dried solid. Molar ratios of the amino acids, after acid hydrolysis, were in the expected range.

The best method found for preparing the linear pentapeptide **9** utilized the reaction of benzyloxy-D-valine *p*-nitrophenyl ester with the triethylammonium salt of **7** to give **8**, followed by hydrogenolysis of that pentapeptide. Compound **8** was obtained in 78% yield after purification on a Sephadex LH-20 column, using methanol as eluent and uv spectra of the fractions to follow the purification. Attempts to condense benzyloxycarbonyl-D-valine with the triethylammonium salt of **7** by other standard coupling methods (use of DCC or Woodward's Reagent K, or *N*-hydroxysuccinimide ester) gave poor results. In particular, the mixed anhydride method gave, as a major product, based on nmr evidence, *O*-(isobutyloxycarbonyl-L-prolylglycyl-

(1) (a) Abbreviations follow the rules of the IUPAC–IUB Commission on Biochemical Nomenclature in *J. Biol. Chem.*, **241**, 2491 (1966), and in *Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967). (b) This work was supported by Public Health Service Research Grant No. 5 R01 CA10571 from the National Cancer Institute. (c) Actinomycin D was so designated by L. C. Vining and S. A. Waksman [*Science*, **120**, 389 (1954)]; subsequently it was found to be identical with actinomycin C_1, so identified by H. Brockmann and H. Gröne, *Naturwissenschaften*, **41**, 65 (1954).

(2) (a) H. Brockmann and H. Lackner, *Naturwissenschaften*, **47**, 230 (1960); (b) *ibid.*, **48**, 555 (1961); (c) *ibid.*, **51**, 435, 384 (1964); (d) *Chem. Ber.*, **100**, 353 (1967); (e) *ibid.*, **101**, 1312 (1968); (f) H. Brockmann, H. Lackner, R. Mecke, G. Troemel, and H.-S. Petras, *ibid.*, **99**, 717 (1966).

(3) (a) J. Meienhofer, *J. Org. Chem.*, **32**, 1143 (1967); (b) *Experientia*, **24**, 776 (1968); (c) *J. Amer. Chem. Soc.*, **92**, 3771 (1970).

(4) Recently, after our yet-unpublished work had been completed, a similar approach was reported by E. Atherton, R. P. Patel, and J. Meienhofer, XXIIIrd IUPAC Meeting, Boston, Mass., 1971.

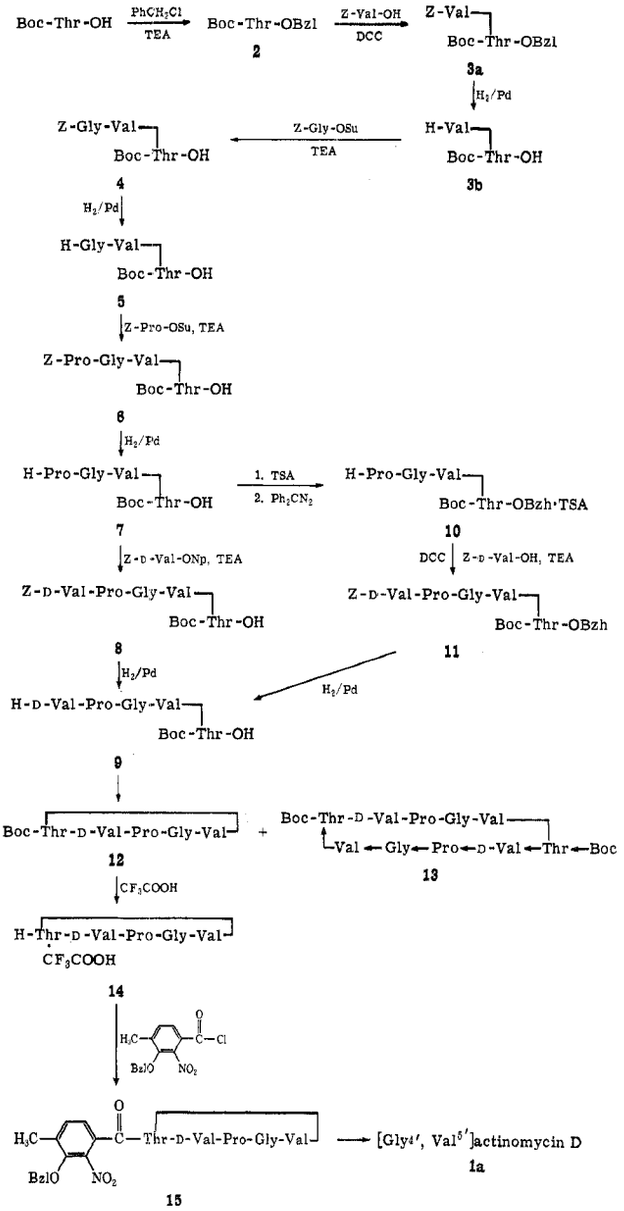
(5) We thank Dr. J. Meienhofer for sending us a preprint[3b] and also for details of preparation of *O*-(L-*N*-methylvalyl)-*N*-*tert*-butyloxycarbonyl-L-threonine and a sample for comparison with the identical product which we had prepared by DCC condensation.

(6) E. Baer and F. Eckstein, *J. Biol. Chem.*, **237**, 1451 (1962).

(7) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).

SCHEME I

SYNTHESIS OF TETRA-*N*-DEMETHYLACTINOMYCIN[a]



[a] All amino acids are of L configuration unless otherwise noted; DCC = dicyclohexylcarbodiimide; TEA = triethylamine; TSA = *p*-toluenesulfonic acid.

L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine, resulting from "reverse" opening of the anhydride intermediate.

Another approach to **9** was successful but less convenient. The benzhydryl ester **10** was prepared from **7** by the method of Aboderin[8] to give a broadly melting solid. This was coupled with benzyloxycarbonyl-D-valine by the DCC method, affording a syrup that could be purified by preparative thick layer chromatography. Similar results and yields were obtained when benzyloxycarbonyl-D-valine *p*-nitrophenyl ester[9,10] was allowed to react with **10**. The blocked pentapeptide **11** so obtained was a sticky solid that could be converted to **9** by hydrogenolysis.

(8) A. A. Aboderin, G. R. Delpierre, and J. S. Fruton, *J. Amer. Chem. Soc.*, **87**, 5469 (1965).

(9) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).

(10) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

Cyclization of **9** by several different methods was attempted. The presence of cyclic pentapeptide in crude reaction mixtures was readily determined by mass spectroscopy; uncyclized **9** and cyclic decapeptide **13** were not sufficiently volatile to be detected. Thus it was determined that only small amounts of **12** were produced when DCC, alone or with *N*-hydroxysuccinimide, was used. Slightly better yields were indicated when *o*-phenylene chlorophosphite[11] in diethyl phosphite was used, but lower molecular weight, volatile products were also produced. Since this procedure involved heating at 140° for about 5 hr, it is quite likely that cleavage of the *tert*-butyloxycarbonyl group in the slightly acidic medium occurred to some extent.

Crude yields of 30–55% resulted from cyclization[12] of the *p*-nitrophenyl ester of **9** (prepared by reaction of di-*p*-nitrophenylsulfite[13] with the *p*-toluenesulfonate salt of **9**) in pyridine at high dilution (about 0.05%, 5×10^{-4} *M* final concentration), the ester being slowly added over a period of several hours. The crude cyclization product was purified on a Sephadex LH-20 column;[14] only a very small amount of **13** was present; yields of purified **12** were in the 23–47% range.

Removal of the *tert*-butyloxycarbonyl blocking group with trifluoroacetic acid at room temperature proceeded cleanly to give **14**, which was then coupled, in the dark, with 2-nitro-3-benzyloxy-4-methylbenzoyl chloride[15] in the presence of *N*-methylmorpholine.[3b] Purification of the product **15** on Sephadex LH-20 gave a 69% yield of slightly discolored solid from which the analytical sample was obtained.

Tetra-*N*-demethylactinomycin (**1a**) was obtained by catalytic reduction followed by controlled oxidation.[16] The orange-red product, purified by precipitation from ethyl acetate solution with hexane (79%), had uv and ir spectra very similar to those of actinomycin D.

The CD spectra of **1a** in methanol, acetonitrile, and chloroform are very similar (Figure 1). It appeared that in methanol solution there was a concentration dependence, but detailed studies were not carried out. The *position* and *magnitudes* of the envelopes are very much like those in the spectra of actinomycin D in the same solvents, but *reversals* of the signs are observed in each case (Figure 2). This is most strikingly seen in the methanol solutions (Figure 3). In hexafluoroacetone hydrate, the CD spectra of actinomycin D and **1a** were very similar; the sign of the Cotton effects in both spectra were the same as those in the spectra of methanol, acetonitrile, or chloroform solutions of **1a** (Figure 4).[17] Thus, we conclude that **1a** essentially has the same conformation in these four solvents, while actinomycin D has similar conformation in hexa-

(11) M. Rothe and F. Eisenbeiss, *Z. Naturforsch. B*, **21**, 814 (1966).

(12) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

(13) B. Iselin and R. Schwyzer, *ibid.*, **43**, 1763 (1960).

(14) (a) H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jap.*, **42**, 782 (1969); (b) M. Kondo and N. Izumiya, *ibid.*, **43**, 1850 (1970).

(15) B. Weinstein, O. P. Crews, M. A. Leaffer, B. R. Baker, and L. Goodman, *J. Org. Chem.*, **27**, 1389 (1962).

(16) W. G. Hanger, W. C. Howell, and A. W. Johnson, *J. Chem. Soc.*, 496 (1958).

(17) It should be noted that the CD spectrum of actinomycin D in hexafluoroacetone sesquihydrate obtained in these laboratories is somewhat different from that reported by Ascoli,[18] particularly in that his spectrum showed a strong absorption maximum ($\Delta\epsilon$ +30) at about 290 nm, while we found no such absorption.

(18) F. Ascoli, P. De Santis, and M. Savino, *Nature (London)*, **227**, 1237 (1970).

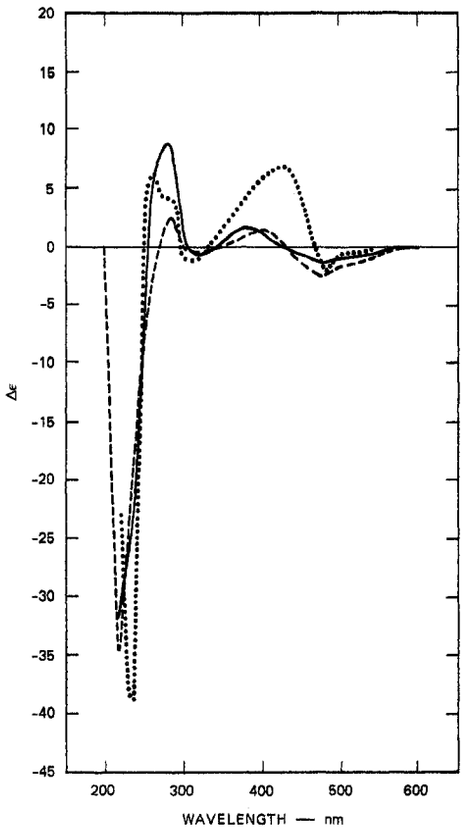


Figure 1.—CD spectra of tetra-*N*-demethylactinomycin: (——) CH_3OH solution, 6×10^{-5} *M*; (····) CH_3CN solution, 3.9×10^{-5} *M*; (- - - -) HFA solution, 9×10^{-5} *M*.

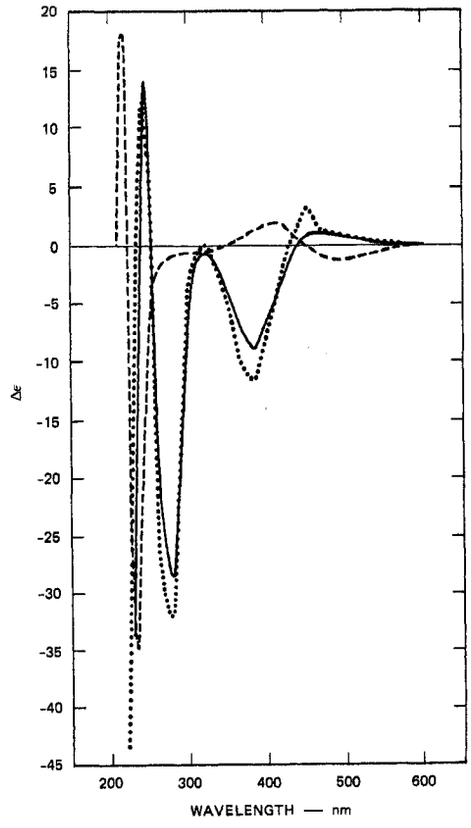


Figure 2.—CD spectra of actinomycin D: (——) CH_3OH solution, 6.5×10^{-5} *M*; (····) CH_3CN solution, 3.7×10^{-5} *M*; (- - - - -) HFA solution, 1×10^{-4} *M*.

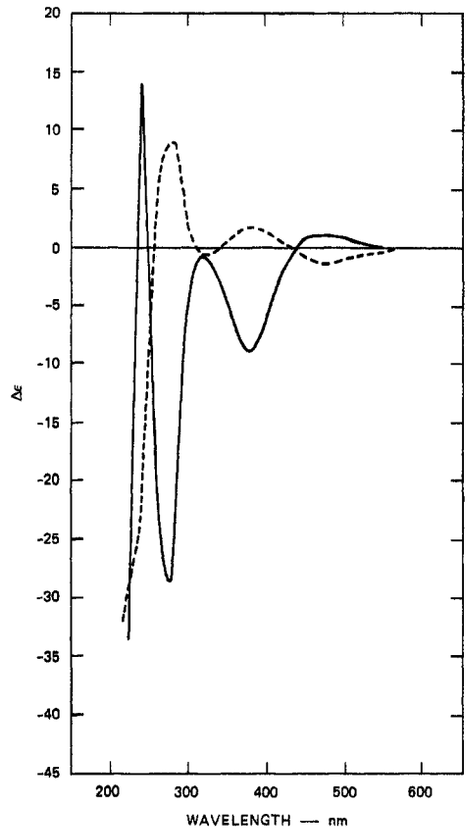


Figure 3.—CD spectra in CH_3OH solution: (——) actinomycin D, 6.5×10^{-5} *M*; (- - - -) **1a**, 6×10^{-5} *M*.

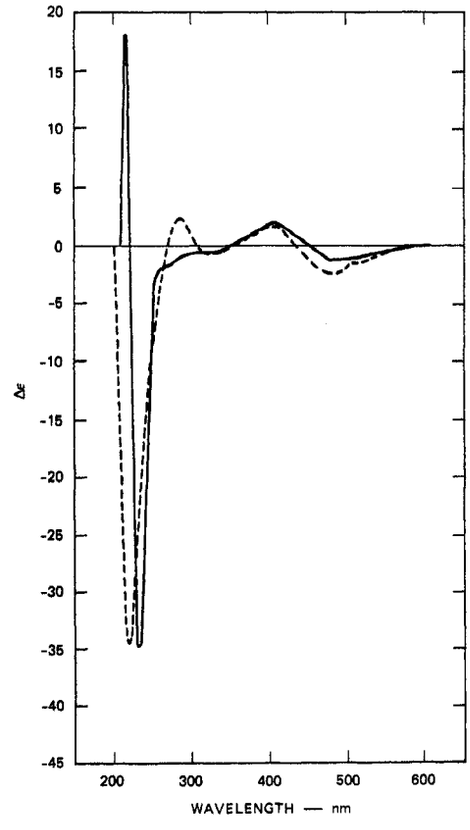


Figure 4.—CD spectra in hexafluoroacetone sesquihydrate solution: (——) actinomycin D, 1×10^{-4} *M*; (- - - -) **1a**, 9×10^{-5} *M*.

fluoroacetone, but a different conformation in methanol, chloroform, and acetonitrile. The increased potential for hydrogen bonding in **1a**, which has two more –NH groups in each peptide lactone ring, may be an important factor.

The nmr spectrum of **15** was very similar to that of (2-nitro-3-methoxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-prolylsarcosyl-*N*-methyl-L-valine (threonine-hydroxyl) lactone, reported by Lackner.[19] However, the nmr spectrum of **1a** was different in many areas from that of actinomycin, which has been variously interpreted.[20-25] Again, these differences reflect increased or changed hydrogen bonding and other conformational changes in the peptide lactone rings.

Antibacterial tests showed **1a** to be inactive against *B. subtilis*, *Staph. aureus*, and a number of other organisms; also, it was noncytotoxic to KB cells in tissue culture tests.

Experimental Section

Methods.—Melting points, uncorrected, were determined on a Fisher-Johns apparatus. Ultraviolet spectra were obtained on a Cary 14 recording spectrophotometer; circular dichroism, on a Durrum-Jasco ORD/UV-5 spectropolarimeter equipped with a Sproul Scientific SS-20 CD modification. Optical rotations were determined at the D line on a Perkin-Elmer 141 polarimeter. Thick layer preparative chromatography was accomplished on Brinkman silica gel HF, 2 mm thick; thin layer chromatography, on silica gel 0.25 mm thick; detection was accomplished by ultraviolet light, by iodine, and by acidic ninhydrin solution. Compounds containing the *tert*-butyloxycarbonyl moiety were detected by first spraying with trifluoroacetic acid, heating at 100° for 5 min, and then spraying with ninhydrin solution and heating again. The following solvent systems were used: (A) methanol–chloroform (3:1); (B) water; (C) 2-butanol–formic acid–water (75:13.5:11.5). Purifications on the Sephadex LH-20 column (2.5 × 72 cm) were accomplished with methanol, flow rate 100 ml/hr, collecting 3-ml fractions; separation was followed by weighing of residues after solvent removal from individual fractions and by thin layer chromatography. Hydrogenolyses were carried out over palladium black (Engelhard) at 1 atm and room temperature. Magnesium sulfate was used as drying agent, and all evaporations were done under reduced pressure (water aspirator). Molecular weight determinations were performed by E. Meier, Department of Chemistry, Stanford University, Stanford, Calif.

***N*-*tert*-Butyloxycarbonyl-L-threonine Benzyl Ester (2).**—Using the method of Baer and Eckstein,[6] *N*-*tert*-butyloxycarbonyl-L-threonine was converted to **2** in 90% yields, mp 40–41°, tlc R_f 0.88 (A). A sample for analysis was recrystallized from ether–petroleum ether (bp 30–60°): $[\alpha]^{21}D$ −19.6° (*c* 1, CH_3OH).

Anal. Calcd for $C_{16}H_{23}NO_5$: C, 62.1; H, 7.49; N, 4.53. Found: C, 62.4; H, 7.60; N, 4.71.

***O*-(Benzyloxycarbonyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine Benzyl Ester (3a).**—To a stirred solution of **2** (10.2 g, 33 mmol) and benzyloxycarbonyl-L-valine (12.55 g, 50 mmol) in dry acetone (30 ml), cooled in ice, was added dry pyridine (3.13 ml, 50 mmol) and DCC (7.25 g, 35 mmol). The mixture was stirred at 0° for 1 hr and then overnight at room temperature. After removal of dicyclohexylurea, which was washed with acetone, the combined filtrates were concentrated; the residual syrup was dissolved in ethyl acetate and washed successively with 1 *M* citric acid solution (twice), dilute $NaHCO_3$ solution, and finally saturated NaCl solution (three times), dried, and concentrated; the crude, syrupy product [19 g, tlc R_f 0.8 (alumina, $CHCl_3$) with trace impurity, mass spectral data confirming the expected structure] was used without further purification in the next step.

***O*-(L-Valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (3b).**—The crude **3a** (19 g) was dissolved in absolute ethanol (200 ml) and subjected to hydrogenolysis; the white solid that separated was dissolved by addition of alcohol. After the catalyst was removed and washed with ethanol, the combined filtrates were concentrated. The solid white product was thoroughly triturated with ether several times, collected on a filter (7.38 g, mp 173–178°), and recrystallized from water (50 ml): yield 6.11 g (60% based on **2**); mp 180.5–181.5°; tlc R_f 0.42 (B), 0.72 (C); $[\alpha]^{21}D$ +42.4° (*c* 1, CH_3OH).

Anal. Calcd for $C_{14}H_{26}N_2O_6 \cdot 3H_2O$: C, 45.2; H, 8.66; N, 7.52. Found: C, 45.4; H, 8.58; N, 7.58.

***O*-(Benzyloxycarbonylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (4).**—To a suspension of **3b** (7.28 g, 22.9 mmol) in dry tetrahydrofuran (50 ml) was added triethylamine (3.2 ml, 22.9 mmol) and then a solution of benzyloxycarbonylglycine *N*-hydroxysuccinimide ester[7] (7.0 g, 22.9 mmol) in dry tetrahydrofuran (25 ml). After the mixture had been stirred overnight at room temperature, the clear solution was concentrated to a syrupy residue, which was dissolved in ethyl acetate and washed with 1 *M* citric acid solution and then with water (three times). It was dried and concentrated, yielding a sticky foam, 11.7 g, which was not purified further. Mass spectral data obtained from the crude methyl ester (prepared by the action of diazomethane[26]) indicated that the expected structure was obtained. A small portion in ether solution was converted to its dicyclohexylammonium salt (96%) and recrystallized from ethanol–ethyl acetate: mp 165–168°; $[\alpha]^{19.5}D$ −9.4° (*c* 0.7, CH_3OH); tlc R_f 0.9 (A), 0.9 (C).

Anal. Calcd for $C_{36}H_{58}N_4O_9$: C, 62.6; H, 8.46; N, 8.11. Found: C, 62.8; H, 8.62; N, 8.16.

***O*-(Glycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (5).**—Hydrogenolysis of **4** in ethanol yielded **5** in 65–75% yield after recrystallization from water: mp 185–187° dec; tlc R_f 0.4 (B), 0.6 (C); $[\alpha]^{22}D$ −1.11 ± 0.3° (*c* 1, CH_3OH).

Anal. Calcd for $C_{16}H_{29}N_3O_7 \cdot {}^1/_2H_2O$: C, 50.0; H, 7.87; N, 10.9. Found: C, 49.7; H, 7.77; N, 10.8.

***O*-(Benzyloxycarbonyl-L-prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (6).**—To a suspension of **5** (6.24 g, 16.6 mmol) in dry tetrahydrofuran (50 ml) was added triethylamine (2.33 ml, 16.6 mmol) and then a solution of benzyloxycarbonyl-L-proline *N*-hydroxysuccinimide ester[7] (5.74 g, 16.6 mmol) in dry tetrahydrofuran. It was stirred overnight at room temperature and then worked up as described for **4**. The crude, solid white product was recrystallized from ethyl acetate (450 ml): yield 6.76 g; mp 183–184.5°; $[\alpha]^{23}D$ −44.5° (*c* 1, CH_3OH); tlc R_f 0.83 (A); second and third crops (1.93 g) raised the total yield to 8.69 g (87%). A small sample was converted to the methyl ester; its mass spectrum confirmed the expected structure.

Anal. Calcd for $C_{29}H_{42}N_4O_{10}$: C, 57.4; H, 6.98; N, 9.25. Found: C, 57.3; H, 6.76; N, 9.17.

***O*-(L-Prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (7).**—Hydrogenolysis of **6** in ethanol gave a very gelatinous material in 83–91% yield; after ether trituration and air-drying, mp 136–141°; tlc R_f 0.2 (B), 0.46 (C). Amino acid analysis (6 *N* HCl, 110°, 22 hr) gave the following molar ratios: valine:proline:glycine:threonine, 1.1:1.1:1.1:1.0.

***O*-(Benzyloxycarbonyl-D-valyl-L-prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (8).**—A solution of benzyloxycarbonyl-D-valine *p*-nitrophenyl ester[9,10] (5.57 g, 15 mmol) in dry tetrahydrofuran (50 ml) was added, with stirring, to a solution of **7** (7.08 g, 15 mmol) and triethylamine (2.10 ml, 15 mmol) in dry tetrahydrofuran (100 ml) and dry dimethylformamide (75 ml); a yellow color developed immediately. After standing for 20 hr at room temperature, the solution was concentrated *in vacuo* to a yellow syrup, which was dissolved in ethyl acetate and washed with 1 *M* citric acid solution and then saturated NaCl solution. Solvent was removed from the dried solution, yielding 12.82 g of a friable foam, which was divided into four portions. Each portion was purified on the Sephadex LH-20 column, using methanol solvent. The fractions were collected and combined according to their ultraviolet spectra; from the four reproducible runs, the material obtained in elution volumes of 208–280 ml totalled 9.0 g.

(19) H. Lackner, *Tetrahedron Lett.*, 3189 (1970).
(20) B. H. Arison and K. Hoogsteen, *Biochemistry*, **9**, 3976 (1970).
(21) F. Conti and P. De Santis, *Nature (London)*, **227**, 1239 (1970).
(22) T. A. Victor, F. E. Hruska, C. L. Bell, and S. S. Danyluk, *Tetrahedron Lett.*, 4721 (1969).
(23) T. A. Victor, F. E. Hruska, K. Hikichi, S. S. Danyluk, and C. L. Bell, *Nature (London)*, **223**, 302 (1969).
(24) P. De Santis, R. Rizzio, and G. Ughetto, *Tetrahedron Lett.*, 4309 (1971).
(25) H. Lackner, *ibid.*, 2221 (9171).
(26) The simplified techniques used in preparing peptide derivatives suitable for mass spectral determinations were suggested by Dr. David W. Thomas, who also obtained and interpreted the spectra.

Crystallization from ethyl acetate yielded 8.0 g, mp 109–115° with softening around 104°, and a second crop of 0.29 g (total yield, 78%): $[\alpha]^{23}D$ −11.7° (*c* 1, CH_3OH); tlc R_f 0.85 (A), 0.88 (C); uv max (CH_3OH) 252 mμ (ε 180), 257 (215), 261 (166), 264 (179), 267 (117).

Anal. Calcd for $C_{34}H_{51}N_5O_{11}$: C, 57.9; H, 7.28; N, 9.93. Found: C, 57.8; H, 7.64; N, 9.98.

***O*-(L-Prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine Benzhydryl Ester *p*-Toluenesulfonate (10).**—The *p*-toluenesulfonic acid salt of **7** was prepared by lyophilizing a solution of **7** (1.89 g, 4 mmol) and *p*-toluenesulfonic acid monohydrate (0.76 g, 4 mmol) in water (15 ml): mp 114–118°; nmr spectrum showed no loss of *tert*-butyloxycarbonyl. The benzhydryl ester (according to the method of Aboderin[8]) was obtained by treating the tosylate salt of **7** (419 mg, 0.65 mmol) with diphenyldiazomethane[27] (188 mg, 0.97 mmol) in dimethylformamide (2 ml) at 50°. After removal of solvent, addition of ether caused separation of an oil which, upon trituration with petroleum ether, solidified to a white solid (320 mg) with a broad melting point below 115°.

***O*-(Benzyloxycarbonyl-D-valyl-L-prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine Benzhydryl Ester (11).**—A solution of **10** (305 mg, 0.38 mmol) in ethyl acetate was treated with triethylamine (0.10 ml, 0.76 mmol) and water (3–4 ml). The ethyl acetate layer was separated, washed with water (three times), dried, and concentrated to yield a friable foam (234 mg), which was coupled with benzyloxycarbonyl-D-valine (113 mg, 0.45 mmol) in methylene chloride (3 ml), using DCC (82 mg, 0.4 mmol). After the mixture had been stirred overnight, it was worked up as described for **3a**; the crude product (330 mg) showed on tlc (ethyl acetate) one spot near the front and one with R_f about 0.4. Attempted purification on a silica column, ethyl acetate eluent, yielded 198 mg of still impure syrup, collected in the first half of the eluate, and 50 mg of sryup, homogeneous on tlc, collected in the second half of the eluate. The impure material was purified on a thick layer chromatographic plate to give 131 mg of sticky product, homogeneous on tlc, R_f 0.4 (ethyl acetate), total yield 181 mg (56%).

***O*-(D-Valyl-L-prolylglycyl-L-valyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (9). A. From 11.**—Hydrogenolysis of **11** (130 mg, 0.15 mmol) in absolute ethanol gave a syrup (106 mg), which solidified upon trituration with ether, yielding a white solid (84 mg, 100%), mp 138–141°. The mass spectrum of a derivatized sample (*tert*-butyloxycarbonyl group removed and the product acetylated and permethylated) showed the presence of the tetrapeptide expected upon cleavage of the ester bond in **9** during permethylation.

Anal. Calcd for $C_{26}H_{45}N_5O_9 \cdot 1^1/_2H_2O$: C, 52.2; H, 8.08; N, 11.7. Found: C, 52.2; H, 8.10; N, 11.4.

B. From 8.—Hydrogenolysis of **8** (4.0 g, 5.67 mmol) in ethanol produced, after removal of ethanol and trituration with ether, a white solid (3.12 g, 96%), mp 136.5–138°, tlc R_f 0.17 (B).

***N*-(*tert*-Butyloxycarbonyl)-L-threonyl-D-valyl-L-prolylglycyl-L-valine (Threonine Hydroxy) Lactone (12).**—A solution of **9** (228 mg, 0.4 mmol) and *p*-toluenesulfonic acid monohydrate (130 mg, 0.4 mmol) in water (5 ml) was lyophilized, leaving a white powder, which was then mixed with di-*p*-nitrophenyl sulfite[13] (130 mg, 0.4 mmol) and dissolved in a solution of dry pyridine (0.07 ml, 0.8 mmol) in ethyl acetate (6 ml). After the solution had been heated (drying tube) at 50° for 3 hr, it was concentrated to a syrup, which was triturated several times with dry ether. A solution of the sticky residue in dry dimethylformamide (3 ml) containing a drop of glacial acetic acid was added dropwise, with stirring, over a 2-hr period to dry pyridine (500 ml) at 55–60°. Stirring at this temperature was continued for 2.5 hr, the pyridine was removed by distillation, and the residue was dissolved in ethyl acetate. After the solution had been washed successively with 1 *M* citric acid solution, dilute $NaHCO_3$ solution, and saturated NaCl solution (until washings were neutral), it was dried and concentrated, yielding a yellow syrup. Purification on Sephadex LH-20 column gave a small amount of material (about 8 mg, decapeptide **13**) in the fractions of elution volume 190–208 ml, and in later fractions (elution volumes 232–259 ml), a much larger amount of **12** (112 mg, 50% yield), mp 118–128°. After two recrystallizations from ethyl acetate–petroleum ether, a first crop (50 mg, mp 128–130°) and a second crop (30 mg, mp 124–128°) were obtained: tlc (acetone–$CHCl_3$, 2:1) R_f 0.72; tlc (CH_3OH–$CHCl_3$, 1:20) R_f 0.40 (R_f 0.0 for **13** in these two systems); $[\alpha]^{22}D$ +15.0° (*c* 1, CH_3OH).

Anal. Calcd for $C_{26}H_{43}N_5O_8$: C, 56.4; H, 7.83; N, 12.7. Found: C, 56.6; H, 8.04; N, 12.7.

Amino acid analysis (6 *N* HCl, 110°, 22 hr) gave the following molar ratios: glycine:valine:threonine:proline, 0.9:1.7:0.8:1.0.

Microbiological assay[28] (4 *N* HCl, 120°, 16 hr hydrolysis) gave the following molar ratios: L-valine:L-threonine:L-proline, 1.0:0.8:1.0.

A sample of **12**, mp 126–130°, obtained in another preparation, was found to have a molecular weight of 546 (theoretical value 553), while **13**, mp 172–175°, had a molecular weight of 1113 (theoretical value 1106).

The mass spectrum of **12** showed a parent peak of *m/e* 553 and various other peaks representing expected fragments upon loss of $-OC_4H_9$, $-C_4H_8-$, and $-CO_2$ and cleavages of the molecule.

L-Threonyl-D-valyl-L-prolylglycyl-L-valine (Threonine Hydroxyl) Lactone Trifluoroacetic Acid Salt (14).—A solution of **12** (60 mg) in trifluoroacetic acid (2 ml), after standing for 1 hr at room temperature, was concentrated to a tan syrup; several triturations with ether yielded a white solid (53 mg), mp 138–141°.

Anal. Calcd for $C_{21}H_{35}N_5O_6 \cdot CF_3CO_2H \cdot {}^1/_2H_2O$: C, 47.9; H, 6.47; N, 12.2 F, 9.89. Found: C, 47.9; H, 6.60 N, 12.1; F, 9.82.

(2-Nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-prolylglycyl-L-valine (Threonine Hydroxyl) Lactone (15).—Light was excluded as much as possible during preparation and purification of **15**, since it is very light sensitive. To an ice-cooled, stirred solution of **14** (1.19 g, 2.1 mmol) in a mixture of dry tetrahydrofuran (5 ml) and dry dimethylformamide (2 ml) was added *N*-methylmorpholine (0.74 ml, 6.4 mmol) followed by a solution of 2-nitro-3-benzyloxy-4-methylbenzoyl chloride (641 mg, 2.1 mmol) in dry tetrahydrofuran (5 ml). The turbid mixture was stirred, cold, for 1 hr and then stored in the refrigerator overnight. After it was diluted with ethyl acetate (100 ml), the reaction mixture was washed successively with 1 *N* HCl (twice) and saturated NaCl solution (four times), dried, and concentrated to yield a crude, friable foam (1.56 g). Purification on the Sephadex LH-20 column yielded 1.04 g (69%) of a pale yellow solid. Precipitation from ethyl acetate solution with hexane gave a white solid (630 mg), mp 131–136°. A sample was reprecipitated for analysis, mp 134–139°.

Anal. Calcd for $C_{36}H_{46}N_6O_{10}$: C, 59.8; H, 6.41; N, 11.6. Found: C, 59.9; H, 6.67; N, 11.4.

Tetra-*N*-demethylactinomycin. 2-Amino-4,6-dimethylphenoxazinone-(3)-1,9-bis[carbonyl-L-threonyl-D-valyl-L-prolylglycyl-L-valine (Threonine Hydroxyl) Lactone] (1a).—A solution of **15** [529 mg, 0.73 mmol, dried for 18 hr at 80° (0.1 mm) over KOH pellets and P_2O_5] in methanol (20 ml) was hydrogenated over 5% palladium on carbon, in the dark. After 2 hr, the mixture was filtered, under nitrogen, through Celite, collecting the filtrate in a flask containing phosphate buffer solution, pH 7.2 (27 ml) and potassium ferricyanide (700 mg, 2.12 mmol). The red-brown mixture was stirred for 20 min, and then diluted with water (80 ml) and ethyl acetate (80 ml). The aqueous layer was separated and extracted three times with ethyl acetate (30-ml portions). The combined ethyl acetate layers were washed successively with 5% $NaHCO_3$ solution (twice), 1 *M* HCl solution (twice), and saturated NaCl solution until washings were neutral. After the solution had been dried and concentrated, the residue was dissolved in ethyl acetate and precipitated with hexane to give 350 mg (79%): mp 213–220°; uv max (CH_3OH) 443 mμ (ε 21,500), 426 (20,900), 237 (38,800), 205 (41,200); $[\alpha]^{28}D$ −433.6° (*c* 0.25, CH_3OH).[29]

Anal. Calcd for $C_{58}H_{75}N_{12}O_{16} \cdot 2H_2O$: C, 56.4; H, 6.69; N, 13.6. Found: C, 56.6; H, 6.39; N, 13.4.

Microbiological assay of tetra-*N*-demethylactinomycin using a number of different organisms in *in vitro* tests carried out at Stanford Research Institute showed it to be totally inactive. Thus, for actinomycin D, the minimal inhibitory concentration for antimicrobial activity (μg/ml), using *B. subtilis*, was 0.5; for tetra-*N*-demethylactinomycin, >1000; using *Staph. aureus*, for actinomycin D, 20; for tetra-*N*-demethylactinomycin, >200.

(27) J. B. Miller, *J. Org. Chem.*, **24**, 560 (1959).

(28) Stereospecific assays for L-threonine, L-valine, and L-proline were done by Shankman Laboratories, Los Angeles, Calif.

(29) This rotation value was obtained on a Durrum-Jasco ORD/UV-5 spectropolarimeter, since the Perkin-Elmer instrument gave irreproducible results, presumably because the solution was so highly colored.

Also cytotoxic tests against KB cells in tissue culture tests carried out under the auspices of the National Cancer Institute showed the tetra-*N*-demethylactinomycin to be completely inactive (at 100 μg/ml). Actinomycin D strongly inhibits KB cells (ID_{50}, 0.002 μg/ml).[3c]

Registry No.—**1a**, 35085-42-8; **2**, 33662-26-9; **3b**, 35085-44-0; **4**, 35085-45-1; **5**, 35085-46-2; **6**, 35085-47-3; **7**, 35085-48-4; **8**, 35085-49-5; **9**, 35085-50-8; **10**, 35085-51-9; **12**, 35085-52-0; **13**, 35085-55-3; **14**, 35085-53-1; **15**, 35085-54-2.

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The Solid-State Dehydrogenation of L-1,4-Cyclohexadiene-1-alanine Hydrate to L-Phenylalanine

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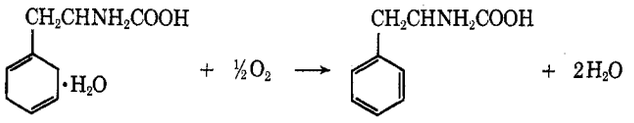
The solid-state dehydrogenation of L-1,4-cyclohexadiene-1-alanine (I) to phenylalanine is shown to be associated with a hydrated form of I. Crystalline, unhydrated, L-, D-, and DL-1,4-cyclohexadiene-1-alanine products are stable, as are the solid cupric and the newly prepared hydrochloride and sodium salts of I. Dehydrogenation requires molecular oxygen. The reaction is accelerated by reducing the pressure in the presence of desiccant, or, at atmospheric pressure, by heating. It takes place at 100° without racemization. The reaction is interpreted to be a transfer of allylic hydrogen to atmospheric oxygen facilitated by aquation.

Organic reactions known to occur in the solid state include largely cyclization and elimination reactions at temperatures below the melting point and radiation-induced decomposition and polymerization reactions. Uncatalyzed, facile dehydrogenation of hydroaromatics at room temperature or below, to our knowledge, has not been described as a solid-state reaction. This communication describes a novel solid-state reaction occurring at room temperature with no catalyst present, the dehydrogenation of L-1,4-cyclohexadiene-1-alanine hydrate to L-phenylalanine (eq 1). In this facile



solid/gas reaction aquation appears to be a means of lowering the activation energy.[1]

L-1,4-Cyclohexadiene-1-alanine (L-DiHPhe, I) is a new and effective antagonist of phenylalanine; it is obtained simply by a one-step Birch reduction of commercial phenylalanine (Phe).[2-4] Soon after its synthesis and properties had been described, L-DiHPhe was identified in three separate laboratories as a new, naturally occurring inhibitor in bacterial sources.[5] Although L-DiHPhe is stable in solution, as a solid at or below room temperature it was observed sometimes to dehydrogenate to Phe.[2] In contrast, DL-DiHPhe was stable in the solid state as well as in solution, which suggested stereospecificity in the solid-state dehydrogenation. Although a crystallization procedure was provided for preparing L-DiHPhe as a stable solid,[2] the side reaction was expected to limit the usefulness of this Phe antagonist for biological purposes.[6] Thus a systematic investigation of the dehydrogenation of DiHPhe was undertaken.

The dehydrogenation product had been identified originally as Phe on the basis of its chromatographic behavior. The product has now been isolated in crystalline form after preparative chromatography on the amino acid analyzer. Its nmr spectrum, optical rotation, and ability to support the growth of *Escherichia coli* 9723f mutant were the same as those of an authentic sample of L-Phe, thus confirming its identity and establishing that it forms without racemization.

The first reproducible observation of dehydrogenation came when material to be dried had been placed in a high vacuum over phosphorus pentoxide at room temperature. In 3 days, as much as 44% transformation to Phe had occurred, whereas another portion of L-DiHPhe left under atmospheric conditions for the same time had undergone only a small change.[7] L-DiHPhe was then observed to separate into two crystalline forms. Stable prisms formed from a dilute solution in 80% ethanol, and unstable needles formed from a hot, saturated solution in 80% ethanol or from a solution in methanol–ethyl acetate. When dissolved at different concentrations in the same solvent (80%

(1) The conversion of 1,4-cyclohexadiene to benzene is carried out at temperatures of 350–500° [see V. A. Mironov and A. A. Akhrem, *Chem. Abstr.*, **68**, 2607f (1968)].

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(4) (a) C. Ressler, D. S. Genghof, C. Lauinger, and M. L. Snow, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **27**, 764 (1968); (b) D. S. Genghof, *Can. J. Microbiol.*, **16**, 545 (1970).

(5) Private communications: T. Yamashita, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, 1968 [see also T. Yamashita, N. Miyairi, K. Kunugita, K. Shimizu, and H. Sakai, *J. Antibiot.*, **23**, 537 (1970)] and G. E. Mallett, Lilly Research Laboratories, 1968. J. P. Scannell, D. L. Pruess, T. C. Demny, T. H. Williams, and A. Stempel, Abstracts, 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970.

(6) Amino acid antagonists of phenylalanine suitable for incorporation into peptides have been needed for efforts to modify hormone activity. *p*-Fluorophenylalanine has been used in this way for the synthesis of bradykinin analogs: E. D. Nicolaides, M. K. Craft, and H. A. DeWald, *J. Med. Chem.*, **6**, 524 (1963).

(7) Observed by Miss C. Lauinger.