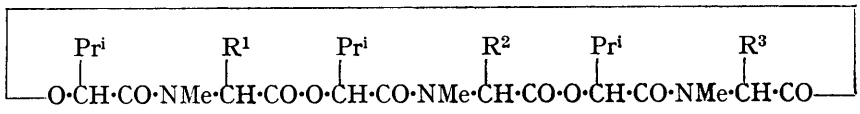
Natural Enniatin A, a Mixture of Optical Isomers containing both *erythro*- and *threo*-*N*-Methyl-L-isoleucine Residues

By **Tapan K. Audhya** and **Douglas W. Russell,*** Biochemistry Department, Dalhousie University, Halifax, Nova Scotia, Canada

Chromatography of a mixture of enniatins from *Fusarium sambucinum* Fuckel yielded a major fraction with the physical properties of synthetic enniatin A. The only amino-acid released by hydrolysis was *N*-methylisoleucine, as a mixture of *erythro*-L and *threo*-L epimers whose ratio showed enniatin A to consist of at least two isomers. Hydrolysis of enniatin in mixed hydrochloric and acetic acids gave completely racemized 2-hydroxyisovaleric acid. The o.r.d. spectra of D-2-hydroxyisovaleric acid and of *N*-methyl-*erythro*-L- and -*threo*-D-isoleucines are recorded.

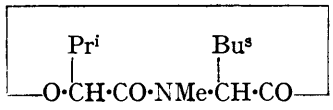
ENNIATINS,[1] cyclohexadepsipeptide antibiotics obtained from mycelia of certain *Fusarium* species, have a regular structure (I) in which residues of D-2-hydroxyisovaleric acid and *N*-methyl-L-amino-acid alternate to form an 18-membered ring bearing six branched alkyl substituents.[2-5] Many enniatins have been synthesized, but only the tris-*N*-methylvaline homologue, enniatin B (I; $R^1 = R^2 = R^3 = Pr^i$), has been obtained pure from a natural source.[6] Enniatin C is the synthetic homologue [7] (I; $R^1 = R^2 = R^3 = Bu^i$); its natural occurrence was inferred from the presence of *N*-methyl-leucine in hydrolysates of natural enniatin mixtures.[8] The isomer (I; $R^1 = R^2 = R^3 = Bu^s$) containing *N*-methyl-*erythro*-isoleucine residues was synthesized,[4,5] to establish the cyclohexadepsipeptide structure of natural 'enniatin A'.[9] The two were indistinguishable by many physical criteria; however, even the purest natural samples yielded some *N*-methylvaline on acid hydrolysis,[9] and their mass spectra had peaks attributable to the 'mixed' enniatins A_1 (I; $R^1 = Pr^i$; $R^2 = R^3 = Bu^s$) and B_1 (I; $R^1 = R^2 = Pr^i$; $R^3 = Bu^s$).[10] We have described the preparation of natural enniatin A, chromatographically freed from enniatins A_1, B_1, B, and C,[11] and now report evidence that this purified material is a mixture of optical isomers.



(I)

The crystalline total enniatin fraction from *F. sambucinum* Fuckel HLX-316 was indistinguishable in m.p., specific rotation, and i.r. spectrum from a sample of synthetic all-*erythro*- (I; $R^1 = R^2 = R^3 = Bu^s$). The mass spectrum of the natural material had a molecular ion peak at *m/e* 681. Paper chromatograms of an acid hydrolysate [12] showed a major ninhydrin-reactive spot with the R_F value of *N*-methylisoleucine, and weaker spots with the R_F values of *N*-methyl-leucine and -valine. By t.l.c.[11] the natural material separated into four components. Column chromatography on silicic acid yielded a major fraction from which natural enniatin A, $C_{36}H_{63}N_3O_9$, was obtained.[11] The latter gave a single spot on t.l.c.; its acid hydrolysates contained neither *N*-methyl-leucine nor *N*-methylvaline, and it was physically indistinguishable from synthetic all-*erythro*- (I; $R^1 = R^2 = R^3 = Bu^s$).[4,5]

Alkaline hydrolysis of natural enniatin A required three equivalents of sodium hydroxide. The acidic product was lactonized to a dioxomorpholine, $C_{12}H_{21}NO_3$ (II), with the same specific rotation as that obtained by



(II)

Plattner and Nager.[9] This lactone was rapidly saponified by 1 equiv. of alkali. Further hydrolysis, with acid, provided D-2-hydroxyisovaleric acid and an amino-acid, $C_7H_{15}NO_2$. Ion-exchange chromatography [13] of the latter gave a major peak with the elution time of *N*-methyl-*erythro*-isoleucine, and a minor one corresponding in position to the *threo*-epimer, in a ratio of *ca.* 3 : 1.

[1] E. Gäumann, S. Roth, L. Ettlinger, Pl. A. Plattner, and U. Nager, *Experientia*, 1947, **3**, 202.
[2] M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryushkin, and V. T. Ivanov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1963, 1055.
[3] P. Quitt, R. O. Studer, and K. Vogler, *Helv. Chim. Acta*, 1963, **46**, 1715.
[4] M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryushkin, and V. T. Ivanov, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1962, 2154.
[5] Pl. A. Plattner, K. Vogler, R. O. Studer, P. Quitt, and W. Keller-Schierlein, *Helv. Chim. Acta*, 1963, **46**, 927.
[6] Pl. A. Plattner and U. Nager, *Helv. Chim. Acta*, 1948, **31**, 665.
[7] Yu. A. Ovchinnikov, V. T. Ivanov, I. I. Mikhaleva, and M. M. Shemyakin, *Izvest. Akad. Nauk S.S.S.R., Ser. khim.*, 1964, 1823.
[8] Pl. A. Plattner and U. Nager, *Helv. Chim. Acta*, 1948, **31**, 2203.
[9] Pl. A. Plattner and U. Nager, *Helv. Chim. Acta*, 1948, **31**, 2192.
[10] A. A. Kiryushkin, B. V. Rozynov, and Yu. A. Ovchinnikov, *Khim. prirod. Soedinenii*, 1968, 157.
[11] T. K. Audhya and D. W. Russell, *Canad. J. Microbiol.*, 1973, **19**, 1051.
[12] D. W. Russell, *J. Chromatog.*, 1960, **4**, 251.
[13] T. K. Audhya and D. W. Russell, *J. Chromatog.*, 1973, **84**, 361.

Identification was substantiated by electrophoresis,[14] and by paper chromatography in a system that separates epimeric pairs of amino-acids.[15] The amino-acid gave a 2,4-dinitrophenyl (DNP) derivative that was photolysed[16] to *N*-methyl-4-nitro-2-nitrosoaniline and 2-methylbutyraldehyde, the latter being isolated as the 2,4-dinitrophenylhydrazone. Both the DNP derivative and the hydrazone slightly depressed the m.p.s of the respective reference samples prepared from *N*-methyl-*erythro*-L-isoleucine.

In 6N-HCl, *N*-methyl-*erythro*-L-isoleucine[17] has $[\alpha]_D$ +46·1°, whereas the *threo*-D epimer has $[\alpha]_D^{22}$ −44·1°,[13] so the value for a 3 : 1 mixture of these two epimers would be +23·8°. The measured value of +40·1°, together with the o.r.d. spectrum, showed both epimers to be >95% in the L configuration at C-2. Thus, either the natural enniatin A contained residues of *N*-methyl-*threo*-L-isoleucine, or this amino-acid had been formed from *erythro*-L residues by partial epimerization at C-3 during hydrolysis. *N*-Methyl-*threo*-L-isoleucine in acid hydrolysates of actinomycins from *Streptomyces antibioticus* is accompanied by only traces of the *erythro*-epimer,[14] and in our experiments *N*-methyl-*erythro*-L-isoleucine was only 2% epimerized under acid-hydrolytic conditions. Nevertheless, 3-epimerization might have occurred during alkaline hydrolysis of the enniatin. Samples of the total enniatin fraction were hydrolysed in three ways, only one of which involved alkali; the ratio of the epimers was the same in all three hydrolysates. (Unexpectedly, the 2-hydroxyisovaleric acid isolated from the hydrolysate prepared with a mixture of acetic and hydrochloric acids was completely racemized.)

We concluded that this natural enniatin A contains residues of *N*-methyl-*threo*-L-isoleucine. As the observed *erythro* : *threo* ratio does not permit an integral number of residues of either epimer, this material must be a mixture of two, three, or four isomers. This conclusion was supported by the results of amino-acid analysis of hydrolysates of fractions comprising the natural enniatin A peak from a silicic acid column. The *erythro* : *threo* ratio was 9·9 : 1 in an early fraction, indicating the presence of all-*erythro*-enniatin A; the ratio decreased progressively to 0·57 : 1 in subsequent fractions, demonstrating the presence of at least two *threo*-residues in another component of the mixture. Unfortunately, we have been unable to obtain samples of natural enniatin A from other sources for comparison.

Cook *et al.*[18] reported that *F. sambucinum* produced a cyclodepsipeptide antibiotic, sambucinin, but now consider that this and the similar antibiotics lateritiin I and II, avenacein, and fructigenin[18] are enniatin mixtures.*

N-methyl-*threo*-L-isoleucine has been obtained from actinomycin hydrolysates,[14] but there has been no previous report of both L epimers in the same group of compounds. It has been suggested[14] that the *threo*-epimer in actinomycin is formed by methylation of *threo*-L-isoleucine derived from the *erythro*-L-epimer, and there is evidence that this epimerization at C-3, perhaps *via* the keto-acid, occurs *in vivo* in man.[19]

The *N*-methyl group of *N*-methylamino-acid residues can be derived from the *S*-methyl group of methionine,[20-23] but it is not known whether the methyl acceptor is a free primary amino-acid or a residue thereof. Sporidesmolide I in *Pithomyces chartarum*[24] is accompanied by small amounts of its unmethylated homologue, sporidesmolide III,[25] suggesting that formation of the cyclodepsipeptide ring precedes methylation.[26] The total enniatin fraction contained traces of material that, unlike enniatins,[27] did not complex with K^+. Hydrolysis yielded valine, leucine, and *erythro*- and *threo*-isoleucine, in proportions similar to those of the corresponding *N*-methyl derivatives in hydrolysates of the total enniatin fraction. Thus the total enniatin fraction may have contained unmethylated analogues: if these were enniatin precursors, it could be inferred that both partial epimerization and cyclization precede *N*-methylation in enniatin biosynthesis.

EXPERIMENTAL

M.p.s were taken with a Kofler hot-stage apparatus and are corrected. I.r. spectra were recorded on a Perkin-Elmer Infracord 137 spectrophotometer, usually as paraffin mulls, and u.v. spectra were measured in ethanol solution with a Carl Zeiss PMQ II instrument. O.r.d. spectra were measured with a Cary 60 spectropolarimeter, using a 0·1 cm cell, and optical rotations were determined in a Bellingham and Stanley model-A polarimeter for the sodium D line. Qualitative and quantitative amino-acid analyses were as previously described.[18] Enniatins were determined as enniatin A by spectrophotometric measurement of the potassium picrate complexes.[28]

Partial Epimerization of N-*Methyl*-erythro-L-*isoleucine*.—The amino-acid[17] gave a single peak on ion-exchange chromatography.[13] A sample (14·5 mg) was heated in a sealed tube at 110° with 6N-HCl (1 ml) for 24 h. Chromatography of the diluted hydrolysate indicated the presence of 2% *threo*-epimer.

Cyclohexylammonium D-2-*Hydroxyisovalerate*.—Prepared

* Professor A. H. Cook, personal communication.

[14] T. Yajima, M. A. Grigg, and E. Katz, *Arch. Biochem. Biophys.*, 1972, **151**, 565.
[15] D. O. Gray, J. Blake, D. H. Brown, and L. Fowden, *J. Chromatog.*, 1964, **13**, 276.
[16] D. W. Russell, *J. Chem. Soc.*, 1964, 2829.
[17] P. Quitt, J. Hellerbach, and K. Vogler, *Helv. Chim. Acta*, 1963, **46**, 327.
[18] A. H. Cook, S. F. Cox, and T. H. Farmer, *J. Chem. Soc.*, 1949, 1022.
[19] B. Halpern and G. E. Pollock, *Biochem. Med.*, 1970, **4**, 352.
[20] A. Sivak, M. Meloni, F. Nobili, and E. Katz, *Biochim. Biophys. Acta*, 1962, **57**, 283.
[21] G. W. Butler, D. W. Russell, and R. T. J. Clarke, *Biochim. Biophys. Acta*, 1962, **58**, 507.
[22] O. Ciferri, A. Albertini, and G. Cassani, *Biochem. J.*, 1964, **90**, 82.
[23] J. E. Walker and D. Perlman, *Biotechnol. and Bioeng.*, 1971, **13**, 371.
[24] D. W. Russell, *J. Chem. Soc.*, 1962, 753.
[25] D. W. Russell, C. G. Macdonald, and J. S. Shannon, *Tetrahedron Letters*, 1964, 2759.
[26] D. W. Russell, *Biochim. Biophys. Acta*, 1972, **261**, 469.
[27] M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, V. K. Antonov, A. M. Shkrob, I. I. Mikhaleva, A. V. Evstratov, and G. G. Malenkov, *Biochem. Biophys. Res. Comm.*, 1967, **29**, 834.
[28] T. K. Audhya and D. W. Russell, *Analyt. Letters*, 1973, **6**, 265.

as described for the L-isomer,[24] the *salt* had m.p. 142—143°, $[\alpha]_D^{22}$ +10·9° (*c* 1·5 in H_2O).

O.r.d. of N-*Methylisoleucines*.—The *erythro*-L-isomer [17] had $[\alpha]_{225}$ +2505° and $[\alpha]_{210}$ 0°, and the *threo*-D-isomer [13] had $[\alpha]_{225}$ −2760°, and $[\alpha]_{211}$ 0° (*c* 0·15 in 0·5N-HCl).

T.l.c. of Enniatins.—Precoated plates of silica gel F-254 (E. Merck) were washed chromatographically in ether and before use were heated at 110° for 2 h. Enniatin samples (0·1 mg) were chromatographed in ethyl acetate–n-hexane–methanol–water, 75 : 200 : 17 : 1,[11] at 18—20°, and spots were detected by exposing the air-dried plates to iodine vapour.

Total Enniatin Fraction of F. sambucinum HLX-316.—The material obtained by processing mycelial extracts to the (90% methanol)–petroleum (b.p. 40—70°) partition stage [11] formed needles (from aqueous ethanol), m.p. 121—122°, $[\alpha]_D^{22}$ −94·1° (*c* 1 in $CHCl_3$), $\nu_{max.}$ 1660 (ester C=O) and 1735 (amide C=O) cm^{-1}, *R*− 0·40, 0·55, 0·63, and 0·68. A single spot, R_F 0·35, was obtained in the t.l.c. system used by Quitt *et al.*[3]

Acid Hydrolysis of the Total Enniatin Fraction.—(i) Samples (227 mg) were hydrolysed (a) in conc. HCl (2 ml) at 110° for 24 h; [9] (b) in conc. HCl (1 ml) + glacial acetic acid (1 ml) at 110° for 24 h; (c) in methanol (1 ml) + N-NaOH (1 ml) at room temperature for 24 h. The solution from (c) was evaporated to dryness *in vacuo*, and the residue was heated in 6N-HCl at 110° for 24 h. Each hydrolysate was evaporated to dryness *in vacuo*, reconstituted in water (2 ml), and extracted with five 4 ml portions of ether; these five extracts were combined, dried (Na_2SO_4), and evaporated to dryness. Each residue, sublimed once *in vacuo*, yielded 2-hydroxyisovaleric acid with the following properties: (a) $[\alpha]_D^{20}$ −19° (*c* 1 in $CHCl_3$), m.p. 59—60°, mixed m.p. with authentic D-isomer, 61—62°; (b) $[\alpha]_D^{20}$ 0°, m.p. 76—78°, mixed m.p. 68—70°; (c) $[\alpha]_D^{20}$ −21°, m.p. and mixed m.p. 62—63°. The ether-insoluble residues were redissolved in water and analysed for *N*-methylisoleucines. The *erythro*:*threo* ratios were (a) 3·2 : 1, (b) 3·4 : 1, and (c) 3·1 : 1.

(ii) The mixture (681 mg), hydrolysed as above in conc. HCl, yielded D-2-hydroxyisovaleric acid (177 mg and 192 mg in two experiments), m.p. 64—65°, mixed m.p. with authentic D-isomer, 64—65°; mixed m.p. with an equal weight of authentic L-isomer, 83—84°; $[\alpha]_D^{22}$ −21·1° (*c* 1·2 in $CHCl_3$), neut. equiv. 120 (calc. for $C_5H_{10}O_3$: 118); cyclohexylammonium salt, plates (from toluene), m.p. and mixed m.p. 142—143°, $[\alpha]_D^{22}$ +11·0° (*c* 1·4 in water). When oxidized with sodium bismuthate [29] the acid afforded isobutyraldehyde 2,4-dinitrophenylhydrazone, m.p. and mixed m.p. 181—182°. I.r. spectra of the acid and its derivatives were indistinguishable from those of authentic samples. The reference sample of D-2-hydroxyisovaleric acid had $[M]_{223}$ −2530°, $[M]_{212}$ 0°, and $[M]_{197}$ +4200°; the isolated acid had $[M]_{223}$ −2470°, $[M]_{212}$ 0°, and $[M]_{197}$ +3800° (*c* 0·1 in 95% ethanol). The ether-insoluble portion of the hydrolysate was evaporated to dryness *in vacuo*; the residue was dissolved in the minimum volume of water, neutralized (pH 7) with triethylamine, and treated with an excess of absolute ethanol. After 18 h at 0° the precipitate was collected, washed with ethanol and ether, and recrystallized from ethanol–water. Amino-acid analysis of the product (401 mg) gave: *N*-methylvaline, 0·215; *N*-methyl-leucine, 0·192; *N*-methyl-*threo*-isoleucine, 0·578; *N*-methyl-*erythro*-isoleucine, 1·857; total 2·842 mmol. In a similar experiment, hydrolysis with conc. HCl and glacial acetic acid (1 : 1) gave, respectively, 0·193, 0·182, 0·491, 1·716; total, 2·582 mmol.

Alkaline Hydrolysis of the Total Enniatin Fraction.—The mixture (681 mg) in methanol (35 ml) was treated with 0·3N-NaOH (15 ml). Portions (5 ml) were withdrawn at intervals and titrated with 0·15N-HCl to pH 8·0; control titrations were performed without the enniatin. Found: alkali consumed after 30 min, 0·85; 60 min, 2·67; 90 min, 2·91; 150 and 210 min, 3·03 mequiv. The reaction of the pooled solutions was adjusted to pH 10; after 18 h, the methanol was removed *in vacuo* and the aqueous residue was adjusted to pH 1·6 (N-H_2SO_4) and continuously extracted with ether for 4 h. The residue obtained by evaporating the dried (Na_2SO_4) ethereal solution was distilled *in vacuo* at 97° and 0·05 mmHg. The distillate in ether was washed with 1% $NaHCO_3$ and water. Two further vacuum-distillations afforded a neutral oil (661 mg), $[\alpha]_D^{22}$ +174·5° (*c* 1·4 in $CHCl_3$).

Natural Enniatin A.—The total enniatin fraction (100 mg) was chromatographed; [11] effluent fractions were analysed for total enniatins as enniatin A, the residues were hydrolysed in acid, and the hydrolysates were analysed for amino-acids. The hydrolysate from fraction 52 contained *erythro*- and *threo*-isoleucine, valine, and leucine, in the ratios 1 : 0·38 : 0·04 : 0·04. Fractions 55—60 from eight runs were pooled and evaporated to dryness *in vacuo*. The residual gum was dissolved in ethanol and the solution was evaporated to dryness. After dissolution and evaporation had been repeated, the residue (400 mg) was crystallized [11] to give natural enniatin A (353 mg) as needles, m.p. 121—122°, $[\alpha]_D^{22}$ −95·1° (*c* 1 in $CHCl_3$). (Under the same conditions, synthetic enniatin A was reported to have m.p. 122—124° [4] and 120—121° [5] and $[\alpha]_D$ −94·5° [4] and −87°.[5]) The natural enniatin A had $\nu_{max.}$ 1660 and 1735 cm^{-1}, R_F 0·63 (Found: C, 63·5; H, 9·3; N, 6·1. Calc. for $C_{36}H_{63}N_3O_9$: C, 63·4; H, 9·3; N, 6·2%). An acid hydrolysate contained no detectable *N*-methyl-leucine, -valine, or -norleucine (see below).

Alkaline Hydrolysis of Natural Enniatin A.—A sample (340·5 mg), treated as above, required 1·48 mequiv. of alkali for saponification and yielded the lactone (II) (310 mg, 91%), $[\alpha]_D$ +178·4° (*c* 1·2 in $CHCl_3$) (lit.,[9] +176·6°), $\nu_{max.}$ (film) 1745 (ester C=O) and 1665 (amide C=O) cm^{-1} (Found: C, 63·4; H, 9·4; N, 6·1. Calc. for $C_{12}H_{21}NO_3$: C, 63·4; H, 9·3; N, 6·2%).

Hydrolysis of the Lactone (II).—The foregoing 2,5-dioxomorpholine (286 mg) in neutral ethanol (2·5 ml) and water (2·5 ml) was titrated slowly with 0·3N-NaOH to pH 8·5 (Found: alkali consumed, 1·25 mmol. Calc. for $C_{12}H_{21}NO_3$: 1·26 mmol). The solution was evaporated to dryness *in vacuo* and the residue was boiled in 6N-HCl (40 ml) for 18 h. When treated as described above, the hydrolysate furnished D-2-hydroxyisovaleric acid (106 mg, 71%) and *N*-methylisoleucine (130 mg, 71%), $[\alpha]_D^{22}$ +40·1° (*c* 1 in 6N-HCl), $[\alpha]_{225}$ +2435°, $[\alpha]_{211}$ 0° (*c* 0·15 in 0·5N-HCl). By i.r. spectrum, paper chromatography,[15] and paper electrophoresis,[14] the amino-acid was indistinguishable from a 1 : 3 mixture of the *threo*- and *erythro*- epimers. Ion-exchange chromatography [13] of a sample (14·5 mg) revealed only the two epimers (24·4 and 74·9 μmol respectively; total 99·3 μmol) (Found: C, 57·8; H, 10·3; N, 9·5. Calc. for $C_7H_{15}NO_2$: C, 57·9; H, 10·5; N, 9·65%).

Degradation of the N-*Methylisoleucine from Natural Enniatin* A.—With 2,4-dinitrofluorobenzene the amino-acid

[29] W. Rigby, *J. Chem. Soc.*, 1950, 1907.

(73 mg) gave a 2,4-dinitrophenyl (DNP) derivative (91 mg), prisms (from CCl_4), m.p. 142—146°; with DNP-*N*-methyl-*erythro*-L-isoleucine [9] (m.p. 148—150°) the mixed m.p. was 146—148°; $[\alpha]_D^{22}$ +488° (*c* 1·2 in $CHCl_3$). Under the same conditions the reference *erythro*-epimer had $[\alpha]_D^{22}$ +502°.

Photolysis of the DNP derivative as described for DNP-*N*-methyl-leucine [16] gave *N*-methyl-4-nitro-2-nitroso-aniline, m.p. and mixed m.p. 166—167°; and 2-methyl-butyraldehyde 2,4-dinitrophenylhydrazone, m.p. 128—131°, mixed m.p. 130—132°, $\lambda_{max.}$ 360 nm (log ε 4·37).

Chromatographic Separation of 2-(N-*Methylamino*)*hexanoic Acids*.—Paper chromatography was done in the solvent system t-pentyl alcohol–acetic acid–water, 20 : 1 : 20 (v/v),[15] as previously described,[13] using *N*-methyl-leucine (MeLeu), *N*-methyl-*threo*- and *erythro*-isoleucine (Me*a*Ile and MeIle respectively), and *N*-methylnorleucine [30] (MeNle). The distances travelled (MeLeu = 100) were Me*a*Ile, 84; MeIle, 87; MeNle, 112 and 113. Ion-exchange chromatography was done as previously reported; [13] elution times in four runs were (i) MeNle, 74; (ii) MeLeu, 66; MeNle, 74; (iii) Me*a*Ile, 58; MeIle, 64: MeNle, 73: (iv) Me*a*Ile, 58; MeIle, 65: MeNle, 74 min. The 'colour constant', H × W/μmol,[13] for MeNle in the four runs was 21·5, 21·4, 22·4, and 21·1.

We thank Drs. M. S. DeWolfe and J. A. Verpoorte for assistance with the ion-exchange chromatography and o.r.d. measurements respectively, Dr. V. T. Ivanov for providing the reference sample of D-2-hydroxyisovaleric acid, and Dr. J. S. Morley for the generous sample of *N*-methylnorleucine. The late Professor M. M. Shemyakin provided the sample of synthetic all-*erythro*- (I; $R^1 = R^2 = R^3 = Bu^s$). This work was supported in part by a grant from the Medical Research Council of Canada. T. K. A. was in receipt of an award from the Killam Trust.

[3/2210 *Received*, 29*th October*, 1973]

[30] J. S. Morley and J. M. Smith, *J. Chem. Soc.* (*C*), 1968, 726.