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THE STRUCTURE OF TRIOSTIN C[1]

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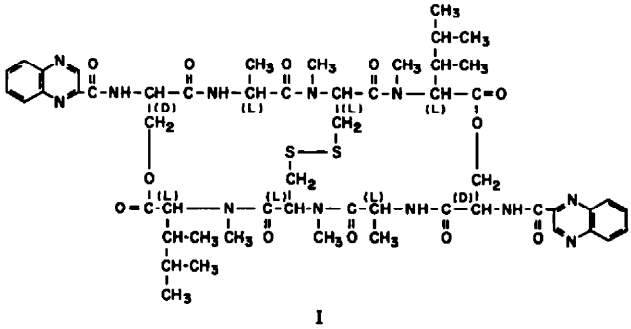
Abstract—Acid hydrolysis of Triostin C proved that the constituents were quinoxaline-2-carboxylic acid (2 moles), D-serine (2 moles), L-alanine (2 moles), N,N′-dimethyl-L-cystine (1 mole), and N,γ-dimethyl-L-alloisoleucine (2 moles). The sequence was proved to be in the order given above and lactone linkage(s) was shown to be present between N,γ-dimethyl-L-alloisoleucine and the hydroxyl group of D-serine. Further evidence indicated the structure I, which differed from Echinomycin in sulphur containing part and N-methylamino acid part.



I

TRIOSTIN C[2] is a major antibiotic product from a streptomycete related to *Streptomyces aureus*.[3] The antibiotic has strong activity against gram positive bacteria, high toxicity to HeLa cells and limited anti-tumor activity against Ehrlich ascites carcinoma.[2,4] It possesses a characteristic solubility property being soluble in chlorinated hydrocarbons, dioxan, formamide, pyridine and acetic acid.

The antibiotic is a practically neutral substance giving no measurable pK_a value. A molecular formula $C_{54}H_{70}O_{12}N_{12}S_2$ (1142), containing four N-methyl groups, is indicated from elemental analysis and mol. wt. determination. The presence of a quinoxaline moiety is suggested by UV absorption (243 mμ and 315–326 mμ) as in the case of Echinomycin.[5–7]

The total acid hydrolysate[8] of Triostin C gave four ninhydrin positive substances on the two-dimensional paper chromatogram in the system n-butanol-acetic acid–water followed by water-saturated phenol. Two of them were serine and alanine. the

[1] Presented, in part, at the 7th Japanese Symposium on the Chemistry of Natural Products Fukuoka, October, 1963.

[2] J. Shōji and K. Katagiri, *J. Antibiotics, Tokyo* Ser. A **14**, 335 (1961).

[3] M. Kuroya, N. Ishida, K. Katagiri, J. Shōji, T. Yoshida, M. Mayama, K. Sato, S. Matsuura, Y. Niinome and O. Shiratori, *J. Antibiotics, Tokyo* **14**, 324 (1961).

[4] S. Matsuura, *J. Antibiotics, Tokyo* **18**, 43 (1965).

[5] R. Corbaz, L. Ettlinger, E. Gäumann, W. Keller-Schierlein, F. Kradolfer, L. Neipp, V. Prelog, P. Reusser and H. Zähner, *Helv. Chim. Acta* **40**, 199 (1957).

[6] W. Keller-Schierlein and V. Prelog, *Helv. Chim. Acta* **40**, 205 (1957).

[7] W. Keller-Schierlein, M. Lj. Mihailovic and V. Prelog, *Helv. Chim. Acta* **42**, 305 (1959).

[8] This result was preliminary reported: H. Otsuka and J. Shōji, *J. Antibiotics, Tokyo* Ser. A **16**, 52 (1963).

third amino acid was positive to *p*-nitrobenzoyl chloride characteristic for N-alkyl amino acid[9] and to sodium nitroprusside–sodium cyanide for disulphide bond,[10] and the last amino acid also was positive to *p*-nitrobenzoyl chloride–pyridine. These amino acids were separated by cellulose column or resin column chromatography. D-Serine and L-alanine were identified from elemental analysis and optical rotational data. The third, sulphur containing amino acid was suggested to be N,N'-dimethylcystine from the elemental analysis. That this suggestion was correct was shown by the synthesis of N,N'-dimethyl-L-cystine through thiazolidine-4-carboxylic acid from L-cystine.[6,11,12] Additional evidence was obtained by desulphurization of this amino acid to N-methylalanine by Raney nickel. Although, this amino acid might be partially racemized during the acid hydrolysis and isolation procedure,[12] positive $[\alpha]_D$ value in 1 N HCl was evidently observed. From that, L-configuration can be proposed to this amino acid.

For the last amino acid, N,β-dimethylleucine structure[8] containing two asymmetric carbon atoms was suggested from the NMR spectrum. The configurational study on this amino acid was carried out by means of NMR spectroscopic examination comparing with reference compounds. Consequently, the stereochemical structure of this amino acid was concluded to be N,γ-dimethyl-L-alloisoleucine.[13]

The presence of a quinoxaline-2-carboxylic acid residue in Triostin C was surmised from its' isolation from Echinomycin[6] whose UV absorption spectrum was nearly identical to that of Triostin C. This compound was not detected in the total acid hydrolysate, but was found in the partial acid hydrolysate and the alkaline hydrolysate. It was isolated by preparative paper chromatography after extraction with ethyl acetate, and was identified by direct comparison with the synthesized one in R_f value, UV and IR absorption. The content of this substance in Triostin C was directly determined to be two moles from the UV absorption of the antibiotic.

When the total acid hydrolysate of Triostin C was analysed by modified Moore and Steins' method[14] by using an automatic amino acid analyser, two moles of serine, alanine, and N,γ-dimethylalloisoleucine and one mole of N,N'-dimethylcystine were determined on the basis of the mol. wt. 1142. Although the alkaline hydrolysate gave a significant amount of ammonia accompanied with almost complete decrease of serine and N,N'-dimethylcystine, no other constitutive fragment except the ones observed in the acid hydrolysate was suspected on the basis of the mol. formula $C_{54}H_{70}O_{12}N_{12}S_2$.

When Triostin C was treated with cold dilute sodium hydroxide aqueous methanol solution, it was converted into an acid (pK_a 5·7 in dioxan–water (1:1, v/v), Neutralization Equiv. 536), shown to have a carboxyl function by absorption band shift[15]; 1737 cm^{-1} (free carboxyl) observed on chloroform solution shifted to 1630 cm^{-1} (carboxylate ion) on chloroform solution added excess triethylamine. The acid (tentatively named alkali-treated Triostin C) showed almost the same UV absorption

[9] S. Edlbacher and Fr. Litvan, *Z. Physiol. Chem.* **256**, 241 (1940); Pl. A. Plattner and U. Nager, *Helv. Chim. Acta* **31**, 2203 (1948).
[10] G. Toennies and J. J. Kolb, *Analyt. Chem.* **23**, 823 (1951).
[11] S. Ratner and H. T. Clarke, *J. Amer. Chem. Soc.* **59**, 200 (1937).
[12] K. Boch and H. T. Clarke, *J. Biol. Chem.* **125**, 275 (1938).
[13] J. Shōji, K. Tori and H. Otsuka, *J. Org. Chem.* in press.
[14] S. Moore and W. H. Stein, *J. Biol. Chem.* **192**, 663 (1951).
[15] A. W. Johnson and J. W. Westly, *J. Chem. Soc.* 1642 (1962).

spectrum as the parent antibiotic, but on acid hydrolysis, gave a trace amount of serine (about 0·1 mole per mole) and two moles of ammonia in addition to the remaining amino acids. The apparent decrease of serine and occurrence of ammonia observed on acid hydrolysis suggested the formation of a dehydroalanine derivative by β-elimination reaction on the O-substituted serine residue caused by the alkali-treatment.[16] In order to prove that this suggestion was correct, mild acid hydrolysis was carried out with alkali-treated Triostin C and 1·8 moles per mole of pyruvic acid was determined as its 2,4-dinitrophenylhydrazone. Additionally, when the dioxan–water solution (1:1, v/v) of alkali-treated Triostin C was boiled at pH 2·0, quinoxaline-2-carboxylamide was released. This indicated that quinoxaline-2-carboxylic acid attached to the N-terminal side of the dehydroalanine residue, whose N—C bond has been proved to be easily cleaved.[17]

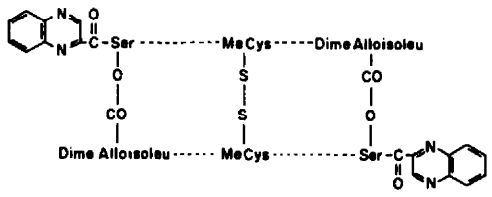
Any DNP amino acid was not derived from alkali-treated Triostin C by 2,4-dinitrophenylation followed by acid hydrolysis, indicating that quinoxaline-2-carboxylic acid attached to N-terminal amino acid. The C-terminal amino acid was determined to be N,γ-dimethylalloisoleucine by Dakin–West degradation.[18] Thus, lactone linkage was suggested to exist in the intact Triostin C, which produce the above described acid, and this was supported by an absorption band at 1745 cm^{-1}.[19,20] That the hydroxyl group of serine was involved in the lactone linkage, was also supported by the fact that chromic acid oxidation[20] on Triostin C did not destroy serine.

A question whether the N,N'-dimethylcystine residue itself was present in Triostin C molecule was resolved as follows. When Triostin C was oxidized by performic acid followed by hydrolysis, N-methylcysteic acid was given in addition to the other amino acids. On the other hand, when the antibiotic was desulphurized by Raney nickel in dioxan–water, about two moles per mole of N-methylalanine were determined on its hydrolysate. A colour reaction detectable for disulphide bond[10]; the reagent consisted of sodium nitroprusside and sodium cyanide in aqueous methanol; was applied without success to the intact antibiotic, presumably because of its insolubility in the reagent. However, after treatment with cold dilute sodium hydroxide for a few minutes, the colour reaction became positive, since lactone ring opening occurred and the solubility increased. A weak absorption band at 410 cm^{-1} observed on Triostin C and alkali-treated Triostin C may be attributable to the disulphide bond.[21]

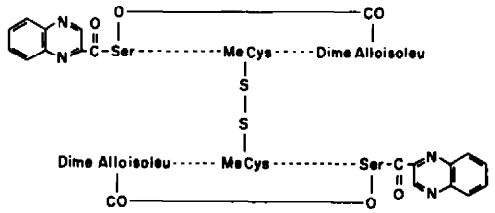
These results obtained up to now indicated a partial structure II or III for Triostin C. However, the formula II was indicated to be correct by mol. wt. determination of desthio-triostin C, which gave almost the same value as that of the parent antibiotic. Thus, the remaining problem to define completely the structure of Triostin C was to elucidate the amino acid sequence between serine and N,γ-dimethylalloisoleucine.

Desthio-triostin C was partially hydrolysed by concentrated hydrochloric acid at

[16] G. Riley, J. H. Turnbull and W. Wilson, *J. Chem. Soc.* 1373 (1957); I. Photaki, *J. Amer. Chem. Soc.* **85**, 1123 (1963); B. Anderson, P. Hoffman and K. Meyer, *J. Biol. Chem.* **240**, 156 (1965).
[17] A. Patchornik and M. Sokolovsky, *J. Amer. Chem. Soc.* **86**, 1206 (1964).
[18] P. A. Turner and G. Schmerzler, *J. Amer. Chem. Soc.* **76**, 949 (1937).
[19] H. Vanderhaeghe and G. Parmentier, *J. Amer. Chem. Soc.* **82**, 4414 (1960).
[20] J. C. Sheehan, H. G. Zachau and W. B. Lauson, *J. Amer. Chem. Soc.* **80**, 3349 (1958).
[21] J. Cymerman and J. B. Willis, *J. Chem. Soc.* 1332 (1951).



II



III

37° for two days, and then n-butanol extraction was carried out in order to separate roughly into chromophore peptide fragments and simple peptide fragments. Then several chromophore peptides, characterized by UV absorption, were separated on two dimensional paper chromatogram in the system n-butanol saturated with aqueous ammonia followed by 1·5 M phosphate buffer, pH 5·0. Two of them were identified as N-(quinoxaline-2-carboxyl)-serine and N-(quinoxaline-2-carboxyl)-seryl-alanine from their constituent amino acids. The remaining two were determined to be N-(quinoxaline-2-carboxyl)-O-(N,γ-dimethylalloisoleucyl)-serine and N-(quinoxaline-2-carboxyl)-O-(N,γ-dimethylalloisoleucyl)-seryl-alanine from their constituent amino acids and release of N,γ-dimethylalloisoleucine by saponification. As simple peptide fragments, seryl-alanine and seryl-alanyl-N-methylalanyl-N,γ-dimethylalloisoleucine were separated by paper chromatography in the system n-butanol–acetic acid–water followed by water–saturated phenol. Moreover, similar acid hydrolysis with Triostin C gave another two peptide fragments consisting of serine, alanine and N,N′-dimethylcystine.

From these results, the structure of Triostin C was concluded to be I. It is evident that there are two series of Quinoxaline Antibiotics; one has a dithiane ring in the molecule like Echinomycin and the other contains a N,N′-dimethylcystine residue, and each series involves several antibiotics differing only in their N-methylamino acid constituents.[8,22] Further studies to clarify the structures of this group of antibiotics are now in progress.

EXPERIMENTAL

M.ps were uncorrected and were determined by a micro m.p. apparatus.

Properties of triostin C

Column chromatography[2] of Triostins was improved in part. Crude crystals (mixture of components C and A; 600 mg) was chromatographed on a silica gel column (Merck, 0·2–0·5 mm) (3·6 × 70 cm). The elution was carried out with $CHCl_3$–MeOH solution, the MeOH concentration of which was increased from 0 to 3% in linear gradient manner. The fractions were detected with optical density at 243 mμ after appropriate dilution with MeOH, and the fractions containing the antibiotics was examined by thin-layer chromatography with Aluminium oxide DF-5 and the solvent (lower layer of ethyl acetate–tetrachloroethane–water; 3:1:3). The fractions containing component

[22] H. Otsuka and J. Shōji, *J. Antibiotics, Tokyo* Ser. A **18**, 134 (1965).

C were concentrated and colourless needles (280 mg) were obtained from $CHCl_3$–MeOH. The homogenity was comfirmed with circular TLC.[23] It had no definite m.p. and gradually degenerated above 260°, λ_{max}^{MeOH} 243 mμ (log ε 4·87), 315–326 mμ (log ε 4·13), $[\alpha]_D^{24}$ −143·9 ± 2° (*c*, 1·121, $CHCl_3$) [Previously reported[3] 210–214°, λ_{max}^{MeOH} 242·5 mμ ($E_{1cm}^{1\%}$ 622), 322·5 mμ ($E_{1cm}^{1\%}$ 115), $[\alpha]_D^{23}$ −133·4°]. (Found: C, 56·46; H, 6·23; N, 14·32; S, 5·21; N—CH_3, 5·50; M.W., 1125 (Barger-Niederl, $CHCl_3$), 1120 (Osmometry, $CHCl_3$). $C_{54}H_{70}O_{12}N_{12}S_2$ requires: C, 56·74; H, 6·13; N, 14·71; S, 5·60; N—CH_3, 5·26%; M.W., 1142.) [Previous analysis[2]: C, 55·40; H, 6·42; O, 16·35; N, 13·86; S, 5·53%.]

Total acid hydrolysis

Triostin C was hydrolysed in 6 N HCl at 105° for 20 hr. The hydrolysate was worked up in a usual way and examined by two-dimensional paper chromatography, giving 4 ninhydrin positive substances and no UV absorbing substance. The R_f values of the substances in the solvent systems n-butanol–acetic acid–water (4:1:2) and water saturated phenol were as follows (for convenience the names of the compounds as subsequently identified are employed): N,N′-dimethylcystine (0·18, 0·84); serine (0·26, 0·37); alanine (0·36, 0·56); N,γ-dimethylalloisoleucine (0·74, 0·92). When developed with a colidine-added MeOH solution of ninhydrin,[24] the serine spot was grayish-purple, alanine was purple, and N,N′-dimethylcystine and N,γ-dimethylalloisoleucine brownish-purple. The later two gave red spots with *p*-nitrobenzoyl chloride–pyridine, indicative of N-alkylamino acids,[9] and N,N′-dimethylcystine afforded also a red spot with sodium nitroprusside–sodium cyanide.[10]

Isolation of hydrolysis products

(a) The total acid hydrolysate from 500 mg Triostin C was concentrated to dryness. The amino acid mixture was extracted with water. The mixture was chromatographed on a cellulose column (Tōyō Roshi, 100–200 mesh; 2·6 × 45 cm) by the solvent n-butanol–acetic acid–water (4:1:2). N,γ-Dimethylalloisoleucine, alanine, serine and N,N′-dimethylcystine were eluted in the order given above.

(b) A similar amino acid mixture from 400 mg Triostin C was chromatographed on a column of Dowex 50 W × 4, 200–400 mesh (1·6 × 130 cm). The elution was carried out with 0·2 M pyridine–formic acid buffer, pH 2·80. N,N′-Dimethylcystine, serine, N,γ-dimethylalloisoleucine, and alanine were eluted but were partially overlapped. Each amino acid was prepared as a crystalline substance in the following manner.

D-*Serine*. The fraction containing serine was decolourized with charcoal and crystallized from water–EtOH as colourless needles, m.p. 220–225° (dec), $[\alpha]_D^{24}$ −14·6 ± 2° (*c*, 0·994, 1 N HCl). (Found: C, 34·07; H, 6·78; N, 12·98. Calc. for $C_3H_7O_2N$: C, 34·28; H, 6·66; N, 13·33%.)

L-*Alanine*. Crystallized from water–EtOH as colourless needles, m.p. 280–282° (dec), $[\alpha]_D^{23}$ +12·1 ± 4° (*c*, 0·626, 1 N HCl). (Found: C, 40·34; H, 7·98; N, 15·72. Calc. for $C_3H_7O_2N$: C, 40·44; H, 7·86; N, 15·73%.)

N,N′-*Dimethyl*-L-*cystine*. This compound was difficult to crystallize, and precipitated in a gel-like state from water–EtOH. Further purification was not carried out, because the available amount was so limited. A preparation showed a m.p. 175–182° (dec), $[\alpha]_D^{26}$ +22·7 ± 2° (*c* 1·049, 1 N HCl), another preparation showed $[\alpha]_D^{25}$ +34·0 ± 2° (*c* 1·022, 1 N HCl) [lit.,[7] m.p. 217–219° (dec), $[\alpha]_D$ +78° (*c* 0·778, 1 N HCl)]. (Found: C, 35·09; H, 6·59; N, 10·26; S, 22·64. Calc. for $C_8H_{16}O_4N_2S_2$: C, 35·82; H, 5·97; N, 10·45; S, 22·88%.) The IR absorption spectrum in Nujol was identical with the synthesized compound, which was prepared as colourless plates, m.p. 215–217° (dec), $[\alpha]_D^{26}$ +58·1 ± 2° (*c* 1·00, 1 N HCl), according to the method described by Keller Schierlein *et al.*[7]

Desulphurization of N,N′-*dimethyl*-L-*cystine*

To 10 mg of the above preparation in 2 ml water, about 300 mg of Raney Ni (W-2) was added. Magnetic stirring was carried out at 45° for 2 hr. The filtrate and water-washings were combined, concentrated, and examined by paper chromatography in the following solvent systems to confirm the same R_f values as those of synthesized N-methyl-D L-alanine. The R_f values in the solvent systems n-butanol–acetic acid–water (4:1:2), water–saturated sec.-butanol, water–saturated t-amyl

[23] To be published.

[24] P. Wollenweber, *J. Chromatog.* 9, 369 (1962).

alcohol, water-saturated benzyl alcohol, and water-saturated phenol were 0·40, 0·22, 0·06, 0·15 and 0·84 respectively.

N,γ-Dimethyl-L-alloisoleucine. After decolourization with charcoal, it crystallized from MeOH–acetone and recrystallized from EtOH as colourless needles, which did not melt nor decompose up to 300°, $[\alpha]_D^{25.5}$ +28·4 ± 2° (*c* 0·937, water) and $[\alpha]_D^{25.5}$ +41·9 ± 2° (*c* 1·049, 5 N HCl). The optical rotational data indicated that this amino acid was in the L-series.[25] (Found: C, 59·80; H, 10·57; N, 8·81. $C_8H_{17}O_2N$ requires: C, 60·37, H, 10·69; N, 8·80%.) The IR absorption and NMR spectra, which were measured in D_2O solution, were identical with those of synthesized N,γ-dimethyl-D L-alloisoleucine.[18]

Quinoxaline-2-carboxylic acid

Triostin C 500 mg was partially hydrolysed in conc. HCl at 37° for 2 days. The hydrolysate was extracted with ethyl acetate, and the water portion was examined for respective amino acids. The ethyl acetate extract was decolourized with charcoal and then chromatographed on paper (Tōyō Roshi No. 50, 40 × 40 cm) with the solvent n-butanol saturated with 3% aqueous ammonia. A UV absorbing substance (R_f 0·25) was extracted from the paper and crystallized from ethyl acetate as colourless needles, m.p. 210–211° (dec), λ_{max}^{MeOH} 243 mμ (log ε 4·53), 315–324 mμ (log ε 3·85). (Found: C, 61·71; H, 3·62; N, 16·08. Calc. for $C_9H_6O_2N_2$: C, 62·07; H, 3·47; N, 16·09%.) The IR absorption spectrum on a KBr disc was identical with that of the synthesized compound, which was prepared by the method of Keller-Schierlein and V. Prelog.[6]

Amino acid analysis

(a) *Acid hydrolysis.* Triostin C in 6 N HCl (4·100 mg/0·5 ml) in a vacuum sealed tube was heated at 105° for 72 hr. The hydrolysate was analysed by a Hitachi Amino Acid Analyser using synthesized N,N'-dimethyl-L-cystine and N,γ-dimethyl-D,L-alloisoleucine as standard compounds. Serine (1·48 moles), alanine (2·02 moles), half N,N'-dimethylcystine (2·16 moles), and N,γ-dimethylalloisoleucine (2·14 moles) were found per mole of Triostin C. When milder conditions were used, a relatively larger quantity of serine[26] was found compared with the other amino acids.

(b) *Alkaline hydrolysis.* Triostin C (2·160 mg) in 5 N NaOH (0·3 ml) was heated at 105° for 20 hr. The hydrolysate was treated with the proper amount of Dowex 50 W (Hydrogen type) to pH 8·2, and then acidified by dil. HCl aq. Amino acid analysis on the above sample gave serine (0·083 moles), alanine (1·98 moles); N,γ-dimethylalloisoleucine (1·67 moles) and ammonia (0·74 moles) per mole of Triostin C. A portion of the hydrolysate was extracted with ethyl acetate, and quinoxaline-2-carboxylic acid was confirmed by paper chromatography and TLC.

Alkali-treated triostin C

Triostin C (100 mg) was suspended in 0·1 N NaOH in 90% aqueous MeOH (6 ml). The mixture was stirred magnetically at 25° for 2 hr. Complete dissolution occurred within 20 min. The solution was diluted with 40 ml water and extracted with ethyl acetate after adjusting the pH to 2·0. The product was then transferred into 5% $NaHCO_3$ aq (15 ml) and re-extracted with ethyl acetate at pH 2·0. The ethyl acetate extract was then washed with water, concentrated, and admixed with n-hexane. The resulting pale yellowish precipitate (93 mg) was dried on P_2O_5, m.p. 159–162°, λ_{max}^{MeOH} 245 mμ (log ε 4·86), 330 mμ (log ε 4·20). (Found: C, 55·65; H, 6·17; N, 13·80; S, 6·11%. M.W., 1300 (Osmometry, $CHCl_3$), 1000–1300 (Barger-Akiya in acetone.) This substance exhibited acidic properties. Titration by using a Radiometer in dioxan–water (1 : 1, v/v) gave a pK_a 5·7 (Neutralization Equiv. 536). The IR absorption spectra indicated the presence of a carboxyl function[15]; 1737 cm^{-1} (free carboxyl) in $CHCl_3$ solution and shifted to 1630 cm^{-1} (carboxylate ion) in $CHCl_3$ solution with triethylamine.

Amino acid analysis on the total acid hydrolysate (6 N HCl, 105°, 24 hr) gave serine (0·065 mole), alanine (1·98 moles), half N,N'-dimethylcystine (2·04 moles), N,γ-dimethylalloisoleucine (2·06 moles), and ammonia (2·32 moles) per mole of the alkali-treated Triostin C, the mol. wt. was assumed to be 1142 for the calculation. The amount of serine deviated between 0·06 and 0·18 (moles per mole) in every preparation.

[25] O. Lutz and B. Jirgensons, *Chem. Ber.* **63**, 448 (1931); P. A. Plattner and U. Nager, *Helv. Chim. Acta* **31**, 2192 (1948).

[26] M. W. Ress, *Biochem. J.* **40**, 632 (1946).

This substance was negative to ninhydrin but positive to sodium nitroprusside–sodium cyanide for the disulphide bond. The 2,4-dinitrophenylation in $NaHCO_3$ aq[27] followed by acid hydrolysis gave no significant 2,4-dinitrophenyl derivatives.

Pyruvic acid

Alkali-treated Triostin C (15 mg) was heated at 100° for 3 hr in 3 N HCl (0·5 ml). To the clear solution 2,4-dinitrophenylhydrazine hydrochloride–methanol solution was added. The resulting yellow crystalline precipitate was collected and examined by TLC using Silica Gel GF 256 (Merck) with the solvent systems $CHCl_3$–MeOH–water (65:30:5) and pet. ether–ethyl formate (13:7) (0·10 ml of propionic acid was added to 100 ml of the above mixture).[28] The R_f values of the yellow spots were 0·48 and 0·42[29] in the former solvent and 0·37 in the latter solvent. These were consistent with those of authentic 2,4-dinitrophenyl hydrazone of pyruvic acid. Quantitative analysis by optical density measurement at 360 mμ indicated 1·8 moles of the hydrazone per mole of alkali-treated Triostin C. The yellow precipitate was then recrystallized from MeOH–water as yellow plate (3·0 mg), m.p. 218–220° (no depression was observed by mixing with the authentic sample). The IR absorption spectrum in Nujol was identical with that of the authentic sample.

When the hydrolysis conditions of alkali-treated Triostin C were more drastic, i.e. increased HCl concentration or time of heating, rapid decrease of pyruvic acid occurred, which has not been reported in the cases of simple dehydroalanine peptides.[17]

Quinoxaline-2-carboxylamide

Alkali-treated Triostin C (108 mg) was dissolved in 20 ml dioxan–water (1:1, v/v). The pH was adjusted to 2·0 and the solution refluxed for 3 hr. The solution was evaporated (red. press.) and extracted with ethyl acetate. The extract was then washed with dil. $NaHCO_3$ aq and concentrated to dryness. When the residue was moistened with ethyl acetate, it gradually crystallized as colourless needles (7·0 mg) and recrystallized from EtOH, m.p. 204·5–205·5° (no depression was observed by mixing with the synthesized compound), λ_{max}^{MeOH} 242 mμ (log ε 4·57), 315–325 mμ (log ε 3·78). The TLC (Silica Gel GF 254, n-butanol saturated with 3% ammonia; R_f 0·61) and the IR absorption spectrum in Nujol were consistent with synthesized quinoxaline-2-carboxylamide.

Dakin-West degradation

Alkali-treated Triostin C (3·0 mg) in acetic anhydride (0·30 ml) and pyridine (0·20 ml) in a sealed tube was heated at 150° for 2·5 hr. The reaction mixture was evaporated; dissolution and evaporation was repeated to remove completely pyridine acetate. It was then hydrolysed in 6 N HCl at 105° for 16 hr and examined by paper chromatography. Alanine, N,N′-dimethylcystine and a trace amount of serine were detected, but no N,γ-dimethylalloisoleucine.

Chromic acid oxidation

Chromic acid (100 mg) was dissolved in pyridine (0·10 ml) and acetic acid (3·0 ml) and the small residue was filtered off. Triostin C (5 mg) was dissolved in the chromic acid solution (0·2 ml) and allowed to stand at 25° for 16 hr. The reaction mixture was then diluted with water (5 ml) and repeatedly extracted with ethyl acetate. The extract was dried and hydrolysed in 6 N HCl at 105° for 16 hr. Amino acid analysis gave an almost equimolar ratio of serine (0·78), alanine (1·00), N,N′-dimethylcystine and N,γ-dimethylalloisoleucine.

Desthio-triostin C

Triostin C (100 mg) was dissolved in dioxan (12 ml) and water (3 ml). Raney Ni (W-2; 1·0 g) was added to the solution, and the mixture stirred at 80–90° for 4·5 hr. The reaction mixture was then filtered and washed with dioxan–water. The filtrate and the washings were evaporated and extracted with ethyl acetate. From the extract, a pale yellowish precipitate (68 mg) was obtained by

[27] F. Sanger, *Biochem. J.* **39**, 507 (1945).

[28] A solvent system for identification of 2,4-dinitrophenylhydrazone of keto-acids: P. Ronkainen, *J. Chromatog.* **11**, 228 (1963).

[29] These double spots were observed with authentic 2,4-dinitrophenylhydrazone of pyruvic acid before recrystallization, and thought to be due to its diastereoisomers.[7]

addition of n-hexane, m.p. 183–186° (dec), λ_{max}^{MeOH} 243 mμ ($E_{1cm}^{1\%}$ 587), 316 mμ ($E_{1cm}^{1\%}$ 105). (Found: S, zero %. M.W., 940 (Osmometry, $CHCl_3$), 1000–1300 (Barger-Akiya in acetone.) Amino acid analysis after hydrolysis (6 N HCl, 105°, 16 hr) gave the following amino acids per mole of this substance (the mol. wt. was assumed to be 1080 for the calculation): serine (1·48 moles), alanine (1·90 moles), N-methylalanine (2·00 moles) and N,γ-dimethylalloisoleucine (1·99 moles).

Oxidized-triostin C

Triostin C (100 mg) was dissolved in formic acid (2 ml). Performic acid reagent[30] (20 ml) was added to the solution, and the mixture allowed to stand at 0° for 16 hr. The mixture was then diluted with water (80 ml) and freeze-dried. The residue was extracted with MeOH and precipitated by addition of ether. An hygroscopic powder (80 mg) was obtained. The amino acid composition was revealed to be serine, alanine, N-methylcysteic acid, and N,γ-dimethylalloisoleucine; the measurement of N-methylcysteic acid deviated to a larger extent, probably because of its strong acidic properties and poor adsorbing qualities on the ion-exchanger.

Partial hydrolysis

(a) Desthio-triostin C (30 mg) was partially hydrolysed in conc. HCl aq (0·20 ml) at 37° for 48 hr. The hydrolysate was dried and dissolved in water (4 ml). This was then extracted with two 4 ml portions of n-butanol to be divided into the butanol layer and the water layer.

When the butanol layer was two-dimensionally chromatographed on paper (Tōyō Roshi, No. 51, 60 × 60 cm) with the solvent system n-butanol saturated with 3% ammonia followed by 1·5 M phosphate buffer, pH 5·0, several dark spots could be separated under UV lamp. The average R_f values in the above solvent systems were as follows; quinoxaline-2-carboxylic acid (1; 0·25, 0·47), N-(quinoxaline-2-carboxyl)-serine (2; 0·15, 0·55), N-(quinoxaline-2-carboxyl)-seryl-alanine(3; 0·13, 0·68), N-(quinoxaline-2-carboxyl)-O-(N,γ-dimethylalloisoleucyl)-serine (4; 0·4–0·6, 0·65), and N-(quinoxaline-2-carboxyl)-O-(N,γ-dimethylalloisoleucyl)seryl-alanine (5; 0·4–0·6, 0·75) (for convenience the structural names consequently elucidated are employed). These had characteristic absorption nearly at 243 mμ (sharp) and 320 mμ (broad) in MeOH. The substances 1, 2 and 3 were negative but 4 and 5 were positive to ninhydrin. The substances (2–5) released respective amino acids involved in their postulated structures described above by the total acid hydrolysis.

When the butanol layer was treated with 0·1 N NaOH in 90% aqueous MeOH at 25° for 30 min followed by the two-dimensional paper chromatography, the spot corresponding to 4 had disappeared and the spot 5 was decreased, and two new spots (R_f values; 0·30, 0·15 and 0·25, 0·35) were present. These two substances (tentatively named 4′ and 5′) had absorptions (244 mμ, 331 mμ and 245 mμ, 320 mμ) in MeOH. The substance 4′ did not give any amino acid by hydrolysis and the substance 5′ gave mainly alanine. On treatment with 0·1 N NaOH in 90% aqueous MeOH, the substances 4 and 5 released N,γ-dimethylalloisoleucine and converted to 4′ and 5′, respectively. From these results, the substances 4′ and 5′ were thought to be quinoxaline-2-carboxyl-dehydroalanine and quinoxaline-2-carboxyl-dehydroalanyl-alanine, respectively.

The water layer was similarly paper chromatographed with the solvent system n-butanol–acetic acid–water (4:1:2) followed by phenol saturated with water. Two ninhydrin positive spots (a) a yellow ninhydrin colour; R_f values of 0·32, 0·45 and (b) a purple colour; R_f values 0·46, 0·75 were separated in addition to the spots corresponding to the constituent amino acids. Amino acid analysis after total hydrolysis revealed that serine and alanine were in (a) and serine, alanine, N-methylalanine and N,γ-dimethylalloisoleucine in (b). The N terminal amino acids proved to be serine from both peptides by DNP method.[31] These results and the information previously obtained indicated that the peptides (a) and (b) were seryl-alanine and seryl-alanyl-N-methylalanyl-N,γ-dimethylalloisoleucine.

(b) Triostin C (30 mg) was hydrolysed in a similar way and followed by the procedure described above. From the water layer two other peptides, whose constituents were serine, alanine and N,N′-dimethylcystine, were isolated; the R_f values were 0·45, 0·35 and 0·60, 0·35 in the solvent systems n-butanol–acetic acid–water (4:1:2) and phenol saturated with water, respectively.

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[30] E. O. P. Thompson, *Biochem. Biophys. Acta* **15**, 440 (1954).
[31] A. L. Leny, *Nature, Lond.* **174**, 126 (1954).