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STRUCTURAL STUDIES ON THE MINOR COMPONENTS OF QUINOXALINE ANTIBIOTICS

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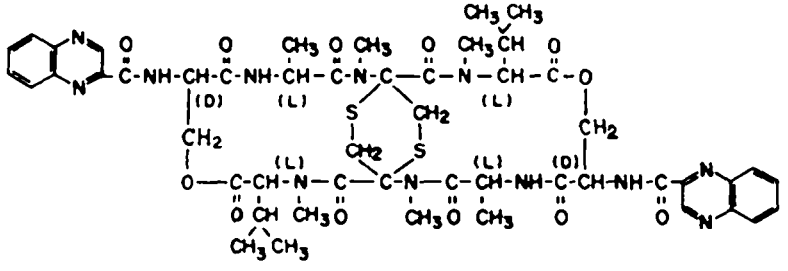
Abstract—Quinomycins A (Echinomycin), B_θ, C, D, B and E and Triostins A, B_θ, C and B were examined by UV and IR absorption spectroscopy and acid hydrolysis. UV absorptions and amino acid analysis of their hydrolysates clarified the following facts as regards their constitutions: quinoxaline-2-carboxylic aicd (2 moles), serine (2 moles) and alanine (2 moles) were common to all of the antibiotics; N,N'-dimethylcystine (1 mole) was present in only the Triostin antibiotics, whereas N-methylamine was found in the hydrolysates of the Quinomycin antibiotics; the differences in each components of both series were found to rest in their N-methylamino acid moieties. The only differences in IR absorptions of the various compounds were in the finger-print and far IR regions. The structures of Quinomycins B_θ, C, D, B and E and Triostins A, B_θ and B have been elucidated on the basis of these data and the published works on Echinomycin and Triostin C.

QUINOXALINE ANTIBIOTICS is a generic name proposed by Kuroya *et al.*[1] for a group of antibiotics containing the quinoxaline ring. The production of Quinomycin complex which contains components A, B^5 and C from two strains related to *Streptomyces aureus* and the isolation of Quinomycin A (Echinomycin) and C have been reported.[2] Shortly after, the production and isolation of Quinomycin B^5 from D,L-isoleucine-added medium was reported.[3] Similarly, the production of Triostin complex which also contains components A, B^5 and C from an another strain related to *Streptomyces aureus* and the isolation of Triostin C has been described.[4] At that time, the structure of Echinomycin had already been elucidated[6–8] as illustrated below.



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

[2] T. Yoshida, K. Katagiri and S. Yokosawa, *J. Antibiotics* Ser. A (Tokyo), **14**, 330 (1961).

[3] T. Yoshida and K. Katagiri, *J. Antibiotics*. Ser. A (Tokyo), **15**, 272 (1962).

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

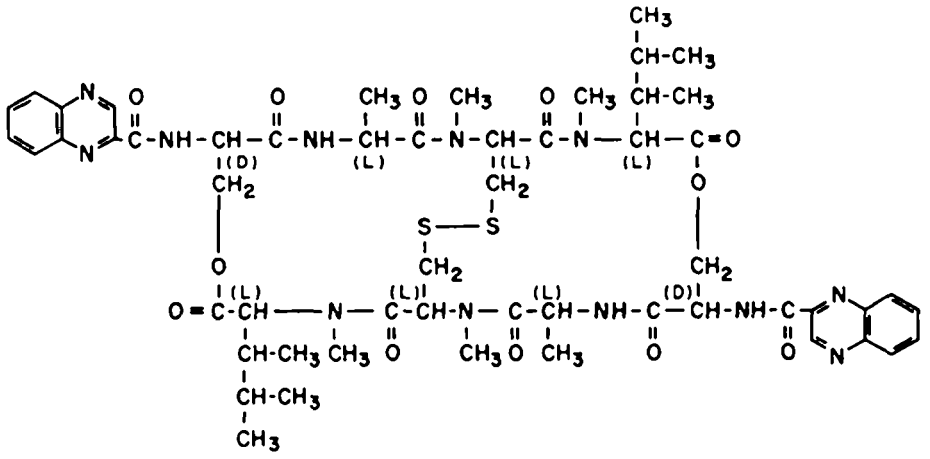
[5] The component B (of both Quinomycin and Triostin antibiotics) produced in natural medium was proved to be different from the component produced in D,L-isoleucine-added medium, in this study. Therefore, the name of the one produced in natural medium was revised to B_θ.

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

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

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

The degradation products of these antibiotics have been studied[9,10] and the structure of Triostin C determined as shown below.



In addition, we studied the structure of other components of this group of antibiotics based on the following hypothesis: (1) Quinomycin antibiotics contain a dithian ring like Echinomycin, on the other hand, Triostin antibiotics contain a N,N'-dimethyl-L-cystine residue as Triostin C. (2) There are several components of Quinomycin and Triostin antibiotics which differ only in their N-methylamino acid moieties. Furthermore, it has been found that two new minor components[12] (named D and E) of both Quinomycin and Triostin antibiotics were produced when the strains were cultured in D,L-isoleucine-added medium.

As a result of the partial revision in nomenclature and the addition of two new components, the components of both Quinomycin and Triostin antibiotics are now named as follows: the three components produced in natural medium are A, B_0 and C, and the five components from D,L-isoleucine-added medium are A, D, B, E and C in the order of increasing R_f value. Since B_0 and B show quite similar chromatographic behaviours with various solvent systems, it has so far not been possible to differenciate these two compounds.

It has been extremely difficult to isolate the respective components and to confirm their homogeneities. A circular TLC on aluminum oxide with a lower layer of ethyl acetate–tetrachloroethane–water (3:1:3) was devised for the separation of the components. This technique was useful for the analysis of the components contained in the various complexes and for the confirmation of the homogeneities of the isolated components (Figs. 1, 2 and 3). Chromatography on silica gel column with the gradient elution technique[10] and on acidic aluminum oxide column with the same solvent system as in the circular TLC was used for the preparative isolation of the components. If the isolation could not be achieved satisfactorily by column chromatography, it was accomplished by preparative circular TLC.

[9] H. Ōtsuka and J. Shōji, *J. Antibiotics*, Ser. A (Tokyo), **16**, 52 (1963).

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

[11] H. Ōtsuka and J. Shōji, *Tetrahedron* **21**, 2931 (1956).

[12] The production of new components has been found by Mr. T. Yoshida and Dr. K. Katagiri of our laboratory. Details will be published elsewhere.

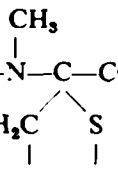
The homogeneities of Quinomycins A, B_0, C, D, B and E, and Triostins A, B_0, C and B thus isolated were confirmed by the circular TLC technique.

The UV absorptions of all these components measured in MeOH were practically identical. The molecular extinctions at two maxima (423 mμ and 315–326 mμ) had almost same values (Table 1), indicating that all components contain 2 moles of quinoxaline-2-carboxylic acid in their molecule.

TABLE 1. UV ABSORPTION OF QUINOXALINE ANTIBIOTICS
(Measured in methanol solutions)

Component		log ϵ^a at 243 mμ	log ϵ^a at 320 mμ
Quinomycins	A	4·81	4·07
	B_0	4·82	4·10
	C	4·82	4·08
	D	4·80	4·09
	B	4·85	4·12
	E	4·82	4·10
Triostins	A	4·85	4·11
	B_0	. .	—[b]
	C	4·85	4·13
	B	4·79	4·01

[a] Mol wts used for calculation were based on the assumed structures.
[b] Not measured

For the amino acid analysis, the antibiotics were hydrolysed with hydrochloric acid and analysed by an automatic amino acid analyser.[13] The N-methylamino acids were isolated before application to the analyser by preparation TLC on cellulose with sec-butanol saturated with water, because their ninhydrin leucine values have been found to be very low.[14] As shown in Table 2, 2 moles of serine and alanine were common to all components; the trace amount of ammonia is presumably derived from the serine residues.[15] One or more moles of methylamine which had been reported to be a degradation product of the  fragment of Echinomycin[7] were found in all the Quinomycin antibiotics. On the other hand, 1 mole of N,N'-dimethylcystine was found in Triostins A and C, whereas the same amino acid was detected qualitatively in Triostins B_0 and B. The N-methylamino acids found in the Quinomycin antibiotics were as follows: 2 moles of N-methylvaline in component A, one mole each of N-methylvaline and N, γ-dimethylalloisoleucine in B_0, 2 moles of N,γ-dimethylalloisoleucine in C, one mole each of N-methylvaline and N-methylalloisoleucine in D, 2 moles of N-methylalloisoleucine in B and one mole each of N-methylalloisoleucine and N,γ-dimethylalloisoleucine in E. The differences found in the N-methylamino

[13] S. Moore and W. H. Stein, *J. Biol. Chem.* **192**, 663 (1951).
[14] M. Ebata, Y. Takahashi and H. Otsuka in press.
[15] M. W. Rees, *Biochem. J.* **40**, 632 (1946).

TABLE 2. AMINO ACID ANALYSES ON QUINOXALINE ANTIBIOTICS
Moles of amino acids per mole of antibiotics

Component		Serine	Alanine	Ammonia	MeAm[a]	DimeCys[b]	MeVal[c]	MeAlloileu[d]	DimeAlloileu[e]
Quinomycins	A	1·38	1·79	0·337	1·13		1·75		
	B_0	1·23	1·78	0·333	1·14		0·957		0·940
	C	1·52	2·01	0·370	1·25				1·83
	D	1·29	1·87	0·358	0·934		0·903	0·854	
	B	1·45	1·85	0·463	1·14			2·09	
	E	1·31	1·95	0·337	1·08			0·834	0·977
Triostins	A	1·46	1·98	0·294		1·05	1·87		
	B_0	+	+	+		+	+		+[f]
	C	1·33	1·89	0·368		1·21			2·14
	B	+	+	+		+		+	

[a] MeAm: Methylamine, [b] DimeCys: N,N′-Dimethylcystine, [c] MeVal: N-Methylvaline, [d] MeAlloileu: N-Methylalloisoleucine, [e] DimeAlloileu: N,γ-Dimethylalloisleucine, [f] Analysed only qualitatively, but N-methylvaline and N,γ-dimethylalloisoleucine were found in equimolar ratio.

acid moieties in the respective components of the Quinomycin antibiotics were also found in the Triostin antibiotics.

The IR absorptions of Quinomycins A, B, E and C and Triostins A and C were all similar (Figs. 4 and 5). The main absorptions and the probable assignments of the

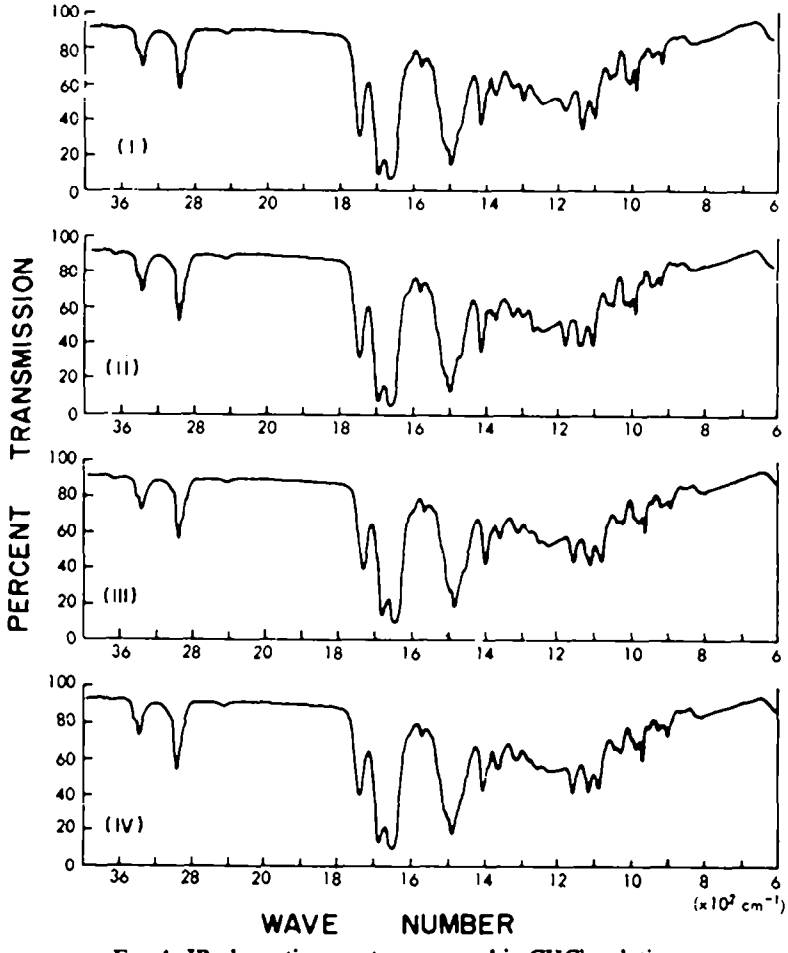


FIG. 4. IR absorption spectra measured in $CHCl_3$ solutions.
(i) Quinomycin A, (ii) Quinomycin B, (iii) Quinomycin E, (iv) Quinomycin C.

Quinomycins are as follows: 3370 cm^{-1} (NH), 2690 (CH_3, CH_2), 1740 (lactone), 1687, 1652 (amide I band), 1502, 1492 (amide II band), 1574 (quinoxaline), 1408 (not assigned), 1395, 1367 (branched methyl),[16] 1322 (not assigned), 1168 (branched methyl),[16] 1125 (lactone), 1098, 1055, 1039, 1011, 997, 980 and 912; the bands in the far IR region as measured with KBr disks are at 620 and 585 (sulphide attached to tertiary carbon[16]). Similarly, those of Triostins are as follows: 3330 (NH), 2960 (CH_3, CH_2), 1746 (lactone), 1672, 1644 (amide I band), 1504, 1492 (amide II band), 1572 (quinoxaline), 1408 (not assigned), 1395, 1370 (branched methyl), 1325 (not assigned), 1165 (branched methyl), 1125 (lactone), 1012, 995, 980 and 911; in the far IR region the bands are at

[16] L. J. Bellamy, *The Infrared Spectrum of Complex Molecule* p. 25 and p. 350. Methuen, London (1958).

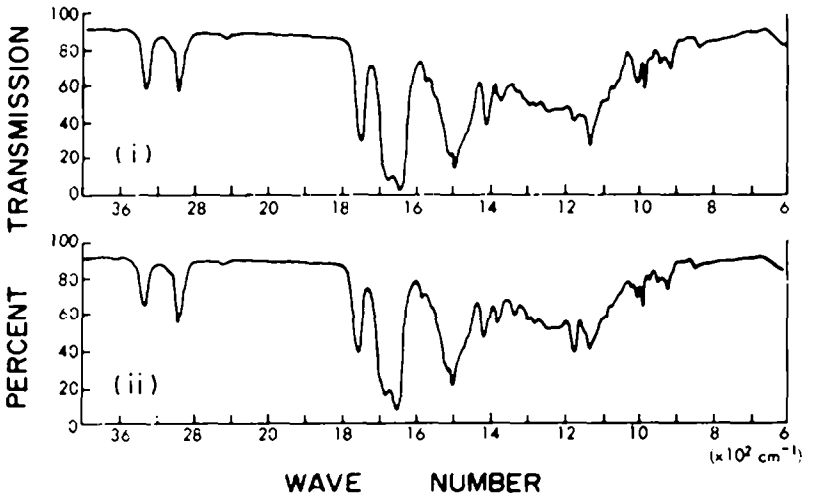
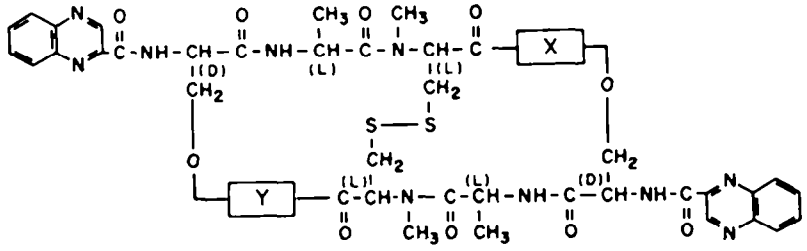


FIG. 5. IR absorption spectra measured in $CHCl_3$ solutions.
(i) Triostin A, (ii) Triostin C.

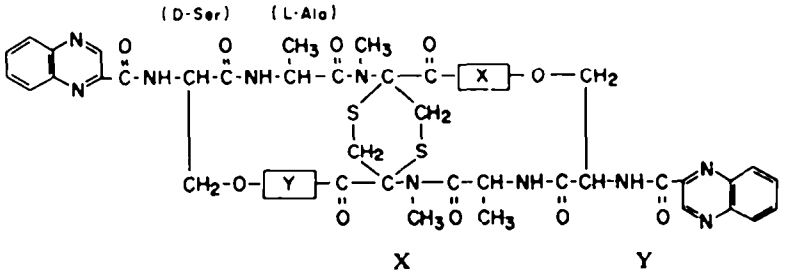
590 (disulphide, C–S),[18] 517, 487, 467, 435 and 410 (disulphide, S–S). The similarity in the IR and far IR absorptions of Quinomycins A, B, E and C indicated that their structural skeletons were very similar. Although Quinomycins D and B_0 were not measured, it is reasonable to consider that the similarity is true for all the Quinomycin antibiotics. The triostin antibiotics are also similar as judged from the spectra of A and C, and that of the complex containing B_0 reported in the previous paper.[4]

From these data, a close similarity was observed in all components of the Quinomycin antibiotics. The only differences in the respective components of the Quinomycin antibiotics was shown in N-methylamino acid moieties. Therefore, the structures of Quinomycins B_0, C, D, B and E are proposed as follows:



	X	Y
Echinomycin (Quinomycin A)	MeVal	MeVal
Quinomycin B_0	MeVal	DimeAlloileu
Quinomycin C	DimeAlloileu	DimeAlloileu
Quinomycin D	MeVal	MeAlloileu
Quinomycin B	MeAlloileu	MeAlloileu
Quinomycin E	MeAlloileu	DimeAlloileu

Similar consideration allows one to derive the following structures for Triostins A, B_0 and B:



	X	Y
Triostin A	MeVal	MeVal
Triostin B_0	MeVal	DimeAlloileu
Triostin C	DimeAlloileu	DimeAlloileu
Triostin B	MeAlloileu	MeAlloileu

EXPERIMENTAL

M.ps are uncorrected and measured by a micro m.p. apparatus. UV absorption spectra were recorded with a Perkin-Elmer 202 UV-visible spectrophotometer, and the intensities at 243 mμ and 320 mμ listed in Table 1 were measured by a Hitachi photoelectric spectrophotometer. IR absorption spectra were recorded with a JASCO Model DS-201B spectrophotometer.

Circular thin-layer chromatography. Aluminum oxide GF_{254} (Merck) with a lower layer of AcOEt–sym-tetrachloroethane-water (3:1:3, v/v)[17] was used. A thin layer plate (20 × 20 cm) with a hole (2 mm diameter) in the center was prepared; the aluminum oxide was mixed with 0·1N HCl to prevent cleavage of the lactone linkage of the antibiotics by the basicity of the adsorbent. The samples were applied as narrow zones on a small circle around the center hole. A cotton wick was inserted through the hole, the plate was placed on a petri dish containing the solvent with the tip of the wick dipping in the solvent, and the entire set-up was settled in a saturating chamber. The developed chromatogram was detected under UV-lump (Mineral light, made by Ultra-Violet Prod. Inc.) and recorded[18] on a print paper of a letter copying machine (Quick Copy, made by Fuji Photo Film Co., Ltd.) by exposure to the UV-light instead of the usual light. The results on Quinomycin and Triostin complexes and the isolated components are illustrated in Figs. 1, 2 and 3.

Isolation of the antibiotics

Quinomycins A, B_0 and C. A Quinomycin complex (134 mg) prepared from the culture in 2% glucose Waksman medium was chromatographed on acidic aluminum oxide column (Woelm, column size; 1·6 × 50 cm) with the same solvent as in the circular TLC. The absorbent was washed with MeOH to remove UV absorbing substances and dried before use. The eluate was examined by UV absorption at 243 mμ in appropriately diluted MeOH solutions and by circular TLC. Components C, B_0 and A were eluted in this order and the respective fractions were concentrated to dryness. By reprecipitation from $CHCl_3$-MeOH, Quinomycin C (42 mg), B_0 (6 mg) and A (23 mg) were obtained as colorless powders.

Quinomycin B. A quinomycin complex prepared from 2% glucose Waksman medium containing 100 mcg/ml of D,L-isoleucine was shown to contain Quinomycin B as the main product. The complex (70 mg) was chromatographed in the manner as described above (column size; 1·0 × 40 cm) to give Quinomycin B (37 mg).

Quinomycins D *and* E. Quinomycin complex prepared from 2% glucose Waksman medium containing 50 mcg/ml of D,L-isoleucine was shown to contain components A, D, B, E and C (Fig. 2). The complex (220 mg) was chromatographed in the same manner (column size; 1·6 × 50 cm) and the eluates were fractionated into aliquots of 10 g. Fractions 22, 23 and 24 contained component E

[17] G. Cassami, A. Albertini and O. Ciferri, *J. Chromatog.* **13**, 238 (1964).
[18] J. Shōji, *J. Chromatog.*, in press.

only, from which Quinomycin E (40 mg) was obtained. Fractions 28, 29 and 30 contained components B and D, while fractions 31, and 32 contained components D and A. These fractions were subjected to preparative circular TLC. The zone corresponding to component D was extracted with $CHCl_3$—MeOH, and 4 mg of Quinomycin D was obtained.

All these preparations were confirmed to be homogeneous by circular TLC (Figs. 1 and 2). The m.ps. were as follows: Quinomycin A 218–221° (dec), Quinomycin B 215–219° (dec), Quinomycin E 215–219° (dec), Quinomycin C 215–218° (dec).

Triostins A, B_0 *and* B. In the previous experiments[10] for the isolation of Triostin C from Triostin complexes by silica gel column chromatography, the fractions containing components A, B_0 and B had been pooled respectively. Triostin B_0 was produced in 2% glucose Waksman medium as a minor product, and B in the medium containing 50 mcg/ml of D,L-isoleucine. A preparation (70 mg) that contained mainly Triostin A was rechromatographed on a silica gel column (Merck, 0·2–0·5 mm, column size; 1·8 × 70 cm) with $CHCl_3$—MeOH, the MeOH concentration of which was increased from 0 to 2% in the linear gradient manner. The fractions shown to contain only component A by circular TLC were collected and concentrated to dryness. The residue was recrystallized from $CHCl_3$—MeOH, and 46 mg of Triostin A was obtained as colorless needles, m.p. 245–248° (dec), $[\alpha]_D^{28.5}$ −157 ± 2° (*c* = 0·970, chf.) (Found: C, 56·27; H, 5·97; N, 15·15; S, 6·31; MW, 1035 (Osmometry, chf.) $C_{56}H_{69}O_{12}N_{13}S_2$ requires: C, 55·25; H, 5·71; N, 15·47; S, 5·89%; MW, 1086).

Preparations containing components B_0 and B were subjected to preparative circular TLC, upon which a few mg of Triostins B_0 and B were obtained in the manner described above.

Total acid hydrolysis

Quinomycins A, B_0, C, D, B and E, and Triostins A, B_0, C and B were compared in parallel runs. A few mg of each compound was hydrolysed with constant boiling HClaq at 105° for 40 hr. After removing the HCl, a portion (about 0·25 μmoles) of the hydrolysate was analysed for serine, alanine and methylamine by a Hitachi automatic amino acid analyzer. For analysis of the N-methylamino acids, another portion (2·5–5·0 μmoles) was applied to a cellulos TLC with sec-butanol saturated with water. N-Methylvaline, N-methylalloisoleucine and N,γ-dimethylalloisoleucine could be separated from serine and alanine, as these N-methylamino acids exhibited relatively high R_f values. The N-methylamino acids were detected by iodine vapour[10] and extracted with water–MeOH (1 : 1 v/v) slightly acidified with HCl. The recoveries of N-methylamino acids were about 80% in this procedure, as checked with authentic compounds. The extract was applied to an automatic amino acid analyser and the content was calculated. In Triostins, additional two runs (about 2·0 and 1·5 μmoles respectively) were made for N,N′-dimethylcystine and ammonia. Triostins B_0 and B were analysed only in a qualitative manner due to lack of sufficient amount of samples. These results are listed in Table 2.

Acknowledgements—The authors are indebted to Dr. M. Ebata for his help in the amino acid analysis by the automatic amino acid analyser.

[10] G. C. Barrett, *Nature, Lond.* **194**, 1171 (1962).