Thermal Degradation of Actinomycins to Dioxopiperazines

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The dioxopiperazines resulting from thermal degradation of several actinomycins were identified by g.l.c. comparison with synthetic compounds, providing a convenient approach to amino-acid sequence determination. In a comparison of relative yields of various dioxopiperazines, different degradative pathways were apparent at different temperatures.

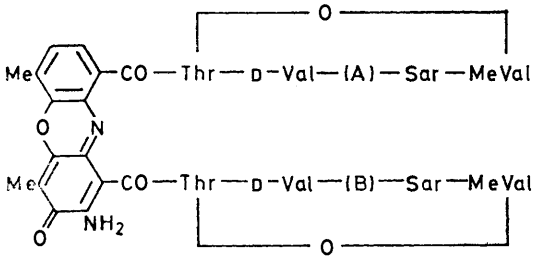
THERMAL degradation of peptides to cyclic dipeptides (dioxopiperazines) can be conveniently investigated by pyrolysis–g.l.c.[1] Application of this technique to several actinomycins (1a—d) has been reported.[2] The peaks observed on pyrograms of actinomycins were identified by comparison of their retention times (Table 1) with those of the dioxopiperazines derived from every possible pair of amino-acids (excluding threonine) in the actinomycin under study. The actual pyrograms [2] and some related g.l.c. studies [3] have been published. The dioxopiperazines were synthesised by standard procedures involving cyclisation of dipeptides or their esters. *cyclo*-(Valylpipecoloyl) and *cyclo*-(valyl-*N*-methylvalyl) were separated into their diastereoisomers by silica gel chromatography. Several dioxopiperazines of *N*-methyl amino-acids were prepared by methylation using methods described previously.[4]

The identification of the products in the degradation of actinomycin D was confirmed by g.l.c.–mass spectrometry, involving comparison of the mass spectra of the synthetic and actinomycin-derived dioxopiperazines (Table 2). Mass spectrometry was also used by Bodanszky *et al.*[5] to identify dioxopiperazines produced by pyrolysis of an amphomycin fragment. Recently, Johnstone *et al.*[6] have utilised g.l.c.–mass spectrometry to identify dioxopiperazines from degradation of a pentapeptide and its Edman-derived tetrapeptide.

The yields [2] of the various dioxopiperazines (Val-Pro > Pro-Sar > Sar-MeVal) produced by pyrolysis of actinomycin D are of interest in connection with the

[1] A. B. Mauger, ' Chemistry and Biology of Peptides ' (Proc. 3rd Amer. Peptide Symp.), ed. J. Meienhofer, Ann Arbor Science Publishers, 1972, p. 691.
[2] A. B. Mauger, *Chem. Comm.*, 1971, 39.
[3] A. B. Mauger, *J. Chromatog.*, 1968, **37**, 315.
[4] A. B. Mauger, R. B. Desai, I. Rittner, and W. J. Rzeszotarski, *J.C.S. Perkin I*, 1972, 2146.
[5] M. Bodanszky, A. A. Bodanszky, C. A. Ralofsky, and R. G. Strong, *J. Antibiotics*, 1971, **24**, 294.
[6] R. A. W. Johnstone, T. J. Povall, and J. D. Baty, *J.C.S. Chem. Comm.*, 1973, 392.

mechanism of the degradation. In peptides which possess a free terminal amino-group, the principal degradative pathway is obvious [7] and the dioxopiperazine formed



(I)

a (Actinomycin D = IV); A = B = Pro [a]
b (Actinomycin C_3); A = B = Pro; D-Val replaced by D-*a*-Ileu [b]
c (Actinomycin II); A = B = Sar [c]
d (Actinomycin Pip 2); A = B = pipecolic acid [d]
e (Actinomycin III); A = Pro, B = Sar or *vice versa* [e]

[a] E. Bullock and A. W. Johnson, *J. Chem. Soc.*, 1957, 3280. [b] H. Brockmann, G. Bohnsack, B. Franck, H. Gröne, H. Muxfeldt, and C. H. Süling, *Angew. Chem.*, 1956, **68**, 70. [c] A. W. Johnson and A. B. Mauger, *Biochem. J.*, 1959, **73**, 535. [d] J. F. Formica, A. J. Shatkin, and E. Katz, *J. Bacteriol.*, 1968, **95**, 2139.

in highest yield represents the *N*-terminal amino-acid pair.[1] An example related to this investigation is the degradation of D-valyl-L-prolylsarcosine to *cyclo*-(D-valyl-L-prolyl).[8] However, this mechanism is blocked in peptides lacking a terminal amino-group such as the actinomycins, and the observation that overlapping dipeptide sequence is obviously superceded by other processes which are already apparent at 170 °C in solution. These processes presumably involve direct interaction of two peptide bonds when the intermediate peptide bond adopts the necessary *cis*-conformation. Such interaction, which can lead to transpeptidation, is far stronger

fragments are produced indicates that more than one degradative pathway is followed. The possibility that dioxopiperazine formation is preceded by ring opening should be considered. This question is related to the fate of the threonine residues. Thus, cleavage could proceed *via* β-elimination (reaction A; R = chromophore) analogous to the base-catalysed reaction observed in the case of echinomycin.[9] Alternatively, an α-elimination (reaction B) of the kind observed in the thermal degradation of etamycin [10] would not produce ring scission.

When the lactone rings of actinomycin D were opened and the resulting actinomycinic acid was pyrolysed under the same conditions as actinomycin D, the relative yields of the various dioxopiperazines were similar (Table 3). This result supports the possibility of prior ring cleavage. The *C*-terminal carboxy-group could then initiate degradation by reaction with the Pro-Sar peptide bond with formation of *cyclo*-(Sar-MeVal) and subsequent formation of *cyclo*-(Val-Pro). However, in view of the relatively low yield of *cyclo*-(Sar-MeVal) this must represent a minor process at 400 °C. On the other hand, this pathway appears to predominate at lower temperatures in solution. Table 3 gives the relative yields of the various dioxopiperazines derived from actinomycin D during 24 h in dimethylformamide solution at several temperatures. Between 130 and 150 °C, *cyclo*-(Sar-MeVal) and *cyclo*-(Val-Pro) are formed in approximately equimolar amounts, and the yield of *cyclo*-(Pro-Sar) is very low. This result would be expected from the degradative scheme outlined above. Moreover, at 120 °C an incomplete degradation reveals *cyclo*-(Sar-MeVal) as the predominant product and indicates that it is the first dioxopiperazine to be released. It is also noteworthy that below 150 °C very little epimerisation occurs during the formation of *cyclo*-(D-Val-L-Pro).

During pyrolysis at 400 °C, the above degradative

TABLE 1

Identification of dioxopiperazines from degradation of actinomycins by their gas chromatographic * retention times (t_R)

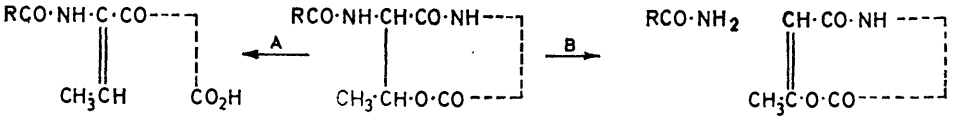
		Actinomycin				
Dioxopiperazine	t_R/min	II	III	IV	Pip 2	IV Meth †
cyclo-(Sar-MeVal) [a]	1.5	+	+	+	+	+
cyclo-(Sar-Sar)	2.0	+	+	−	−	−
cyclo-(Val-MeVal) (*cis*)	3.5	−	−	−	−	−
cyclo-(Val-MeVal) (*trans*)	2.4	−	−	−	−	−
cyclo-(Val-Sar) [b]	3.2	+	+	−	−	−
cyclo-(MeVal-Pip) (*cis*)	4.8	−	−	−	−	−
cyclo-(MeVal-Pip) (*trans*)	3.3	−	−	−	−	−
cyclo-(MeVal-Pro) (*cis*)	3.8	−	−	−	−	+
cyclo-(MeVal-Pro) (*trans*)	3.8	−	−	−	−	+
cyclo-(Pro-Sar) [a]	4.4	−	+	+	−	+
cyclo-(Pip-Sar) [a]	4.5	−	−	−	+	−
cyclo-(Val-Pro) (*cis*) [c]	6.6	−	+	+	−	−
cyclo-(Val-Pro) (*trans*) [d]	9.6	−	+	+	−	−
cyclo-(Val-Pip) (*cis*)	9.0	−	−	−	+	−
cyclo-(Val-Pip) (*trans*)	7.0	−	−	−	+	−

* Column A at 160 °C. † Permethylated actinomycin IV or permethylated tetrademethylactinomycin IV.

[a] Ref. 4. [b] N. A. Poddubnaya and G. I. Lavrenova, *Zhur. obshchei Khim.*, 1958, **28**, 404. [c] A. Butenandt, P. Karlson, and W. Zillig, *Z. physiol. Chem.*, 1951, **288**, 279. [d] I. Z. Siemion, *Org. Magnetic Resonance*, 1971, **3**, 545.

[7] N. Lichtenstein, *J. Amer. Chem. Soc.*, 1938, **60**, 560.
[8] J. Meienhofer, *J. Amer. Chem. Soc.*, 1970, **92**, 3771.
[9] W. Keller-Schierlein, M. L. Mihailovic, and V. Prelog, *Helv. Chim. Acta*, 1959, **42**, 305.
[10] R. B. Arnold, A. W. Johnson, and A. B. Mauger, *J. Chem. Soc.*, 1958, 4466.

in the case of secondary than of primary amino-acids owing to the greater nucleophilicity of an *N*-alkyl than of an NH group.[11] It follows that the ease with which actinomycins are degraded to dioxopiperazines is dependent upon their amino-acid composition. The synthesis [12] of an analogue of actinomycin D, in which the sarcosine and *N*-methylvaline residues were replaced by glycine and valine respectively, provided an opportunity to compare its pyrolysis products with those of actinomycin D. In this case *cyclo*-(Val-Pro) was produced almost exclusively; only a trace of *cyclo*-(Pro-Gly) and no *cyclo*-(Gly-Val) were detected. On the other hand, when this tetrademethylactinomycin was permethylated, subsequent pyrolysis produced all the possible dioxopiperazines (Table 1), the same result being obtained with permethylated actinomycin D.



EXPERIMENTAL

Gas–Liquid Chromatography.—A Glowall 310 gas chromatograph was used, equipped with a flame ionisation detector and glass columns (6 ft × 3.4 mm) with argon as carrier gas at 40 ml min^{-1}; column A: 1.5% EGSP-Z; column B: 3% EGSP-Z, both on Gas Chrom Q (100—120 mesh).

Pyrolysis—Gas Chromatography.—A Hamilton Probe Sampling System (Hamilton Co., Whittier, California) was used. The sample (10—25 μg) of an actinomycin, actinomycinic acid,[13] permethylated [14] actinomycin, or tetrademethylactinomycin [12] was held at 400 °C in the flowing mode using column B at 200 °C. For the determination of yields in the case of actinomycin D, an internal standard, *cyclo*-(Gly-Leu), was mixed with actinomycin D in equimolar amounts prior to pyrolysis, and quantitation was effected by weighing photocopied chromatographic peaks. In two replicate experiments, retention times, peak areas (relative to internal standard), and relative molar detector responses (in parentheses) were: *cyclo*-(Sar-MeVal), 5.5 min, 0.37, 0.38 (1.34); *cyclo*-(Pro-Sar), 11.3 min, 0.50, 0.52 (0.94); *cyclo*-(Val-Pro) (*cis*), 13.8 min, 0.15, 0.17 (1.10); *cyclo*-(Val-Pro) (*trans*), 16.1 min, 0.71, 0.73 (1.22); *cyclo*-(Gly-Leu), 19.7 min, 1.00 (1.00). The calculated yields in mol of dioxopiperazine per mol of actinomycin were: *cyclo*-(Sar-MeVal), 0.28; *cyclo*-(Pro-Sar), 0.54; *cyclo*-(Val-Pro) (*cis*), 0.15; *cyclo*-(Val-Pro) (*trans*), 0.59; *cyclo*-(Val-Pro) (total), 0.74.

G.l.c.–Mass Spectrometry.—An LKB 9000 instrument was

TABLE 2

Mass spectra * of dioxopiperazines: comparison of the synthetic compounds with the degradation products from actinomycin D

	cyclo-(Sar-MeVal)			*cyclo*-(Pro-Sar)			*cyclo*-(Val-Pro) (*trans*)		*cyclo*-(Val-Pro) (*cis*)	
m/e	Synth.	Degn.	*m/e*	Synth.	Degn.	*m/e*	Synth.	Degn.	Synth.	Degn.
184	0.02	0.03	168	0.89	0.80	196	0.03	0.03	0.02	0.03
143	0.08	0.12	140	0.41	0.35	155	0.11	0.12	0.10	0.13
142	1.00	0.99	112	0.46	0.44	154	1.00	1.00	1.00	0.93
141	0.54	0.55	111	0.44	0.45	125	0.47	0.46	0.33	0.31
113	0.93	1.00	83	1.00	1.00	72	0.23	0.23	0.35	0.40
71	0.15	0.23	70	0.69	0.78	70	0.94	0.90	0.95	1.00

* Relative intensites are shown for the molecular ion and five other most prominent ions in each spectrum.

TABLE 3

Relative yields * of dioxopiperazines derived from actinomycin D and actinomycinic acid D under various conditions

	Actinomycin D						Act. acid
Dioxopiperazine	120 °C [a]	130 °C [a]	140 °C [a]	150 °C [a]	170 °C [a]	400 °C [b]	400 °C [b]
cyclo-(Sar-MeVal)	0.56	0.41	0.43	0.44	0.33	0.18	0.22
cyclo-(Pro-Sar)	0.13	0.03	0.05	0.08	0.23	0.34	0.37
cyclo-(Val-Pro) (*cis*)	0.06	0.01	0.06	0.01	0.11	0.10	0.09
cyclo-(Val-Pro) (*trans*)	0.25	0.55	0.46	0.47	0.33	0.38	0.32
cyclo-(Val-Pro) (total)	0.31	0.56	0.52	0.48	0.44	0.48	0.41

* Molar fraction of total. [a] In dimethylformamide solution. [b] Pyrolysis–gas chromatography.

In summary, under relatively mild conditions an orderly sequential degradation occurs which precludes formation of overlapping dipeptide fragments. At higher temperatures, however, other degradative processes occur, leading to overlapping fragments and hence to more complete amino-acid sequence information. These processes are enhanced by the presence of secondary amino-acid residues, and it follows that prior permethylation may be advantageous in the more general application of pyrolysis to sequence determination in peptides.

[11] J. Dale and K. Titlestad, *Chem. Comm.*, 1970, 1403.
[12] C. W. Mosher and L. Goodman, *J. Org. Chem.*, 1972, **37**, 2928.
[13] H. Ziffer, K. Yamaoka, and A. B. Mauger, *Biochemistry*, 1968, **7**, 966.
[14] B. C. Das, S. D. Gero, and E. Lederer, *Biochem. Biophys. Res. Comm.*, 1967, **29**, 212.

employed, equipped with a steel 6 ft × 1/8 in column of 3% EGSP-Z on GasChrom Q (100—120 mesh) at 200 °C. For comparison with the synthetic dioxopiperazines (Table 2) a sample (4 mg) of actinomycin D was heated at 260° and 0.5 mmHg for 1 h and the sublimate was resublimed at 160° and 0.5 mmHg.

Thermal Degradation of Actinomycin D in Solution.—Samples (0.5 mg) of actinomycin D in dimethylformamide (10 μl) were heated in sealed m.p. tubes for 1 h at 120, 130, 150, and 170 °C. Samples (1 μl) of each reaction solution were injected into the gas chromatograph (column B at 190 °C). Relative peak areas were measured and relative (molar) yields calculated (Table 3).

cyclo-(L-*Isoleucyl*-L-*prolyl*).—L-Isoleucyl-L-proline (28 mg) and phenol (0.80 g) were heated (sealed tube) at 170 °C for 3 h. The phenol was removed at 24° and 1 mmHg and the residual solid twice sublimed at 150° and 1 mmHg to give white *needles*, m.p. 130—132° (Found: C, 62.8; H, 8.4; N, 13.15. $C_{11}H_{18}N_2O_2$ requires C, 62.85; H, 8.65; N, 13.35%).

cyclo-(*Valylpipecolyl*) (L,L- *and* L,D-).—Benzyloxycarbonyl-L-valine (1.107 g) in acetonitrile (6 ml) was added to a stirred mixture of methyl DL-piperidine-2-carboxylate hydrochloride (0.833 g) and acetonitrile (15 ml) containing triethylamine (0.7 ml). The mixture was stirred in an ice-bath and dicyclohexylcarbodi-imide (0.861 g) in acetonitrile (5 ml) was added. After 7 h at 24 °C the mixture was worked up as usual and the resulting methyl benzyloxycarbonyl-L-valyl-DL-pipecolate was dissolved in 30% hydrogen bromide in acetic acid (20 ml) and kept at 24 °C for 2 h. After evaporation at 24° and 1 mmHg, the resulting syrup was triturated with dry ether (3 × 50 ml) and the residual methyl L-valyl-DL-pipecolate hydrobromide was dissolved in saturated methanolic ammonia (95 ml). After 40 h at 24 °C, the solution was evaporated and the crude dioxopiperazine was recovered and separated into its diastereoisomers by chromatography on a column of silica gel (Merck; 0.05—0.20 mm) with 10% methanol–chloroform. After sublimation at 170° and 0.2 mmHg and recrystallisation from chloroform–petroleum, cyclo-(L-*valyl*-L-*pipecoloyl*) formed needles (97 mg), m.p. 159—161° (Found: C, 62.8; H, 8.85; N, 13.25. $C_{11}H_{18}N_2O_2$ requires C, 62.85; H, 8.65; N, 13.35%); cyclo-(L-*valyl*-D-*pipecoloyl*) formed needles (147 mg), m.p. 139—141° (Found: C, 62.8; H, 8.9; N, 13.4%).

Methylated Dioxopiperazines. The *cis*- and *trans*-isomers of *cyclo*-(MeVal-Pro) and *cyclo*-(MeVal-Pip) were prepared on a small scale for g.l.c. by methylation of the corresponding isomers of *cyclo*-(Val-Pro) and *cyclo*-(Val-Pip) as described previously.[4,15]

cyclo-(*Valyl*-N-*methylvalyl*).—Phthaloyl-L-valine (1.23 g) and thionyl chloride (1.5 ml) in dry benzene (15 ml) were heated under reflux for 30 min. The mixture was then evaporated, the residual acid chloride was dissolved in dry benzene (10 ml), and *N*-methyl-L-valine methyl ester hydrochloride [4] (0.95 g) was added. The mixture was stirred and triethylamine (1.4 ml) was added over 20 min. After 16 h, the crude phthaloyl-L-valyl-*N*-methyl-L-valine methyl ester was chromatographed over a column of silica gel with 5% ethyl acetate–chloroform. The product was heated in ethanol (24 ml) containing hydrazine (1 ml) under reflux for 1 h. The cyclodipeptide so obtained was separated into diastereoisomers by silica gel column chromatography with 5% methanol–ethyl acetate. After sublimation at 140° and 1 mmHg and recrystallisation from ethyl acetate–petroleum, cyclo-(L-*valyl*-N-*methyl*-L-*valyl*) (240 mg) formed needles, m.p. 72—74° (Found: C, 62.45; H, 9.75; N, 13.4. $C_{11}H_{20}N_2O_2$ requires C, 62.25; H, 9.5; N, 13.2%); cyclo-(L-*valyl*-N-*methyl*-D-*valyl*) (66 mg) formed prisms, m.p. 104—106° (Found: C, 62.2; H, 9.75; N, 13.15%).

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[15] J. R. Coggins and N. L. Benoiton, *Canad. J. Chem.*, 1971, **49**, 1968.