# Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. VII. Synthesis of Ile[8],Orn[10]- and Ala[8],Orn[10]-Eicosapeptides[1]

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**Abstract:** Syntheses are described of two analogs of S-peptide where the arginyl residue, in position 10, has been replaced by ornithine, and the phenylalanyl residue, in position 8, either by isoleucine or alanine. The stereochemical homogeneity of these peptides, *i.e.*, lysylglutamylthreonylalanylalanylalanyllysylisoleucylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine and lysylglutamylthreonylalanylalanylalanylalanyllysylalanylglutamylornithylglutaminylhystidylmethionylaspartylserylserylthreonylserylalanylalanine, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. The enzymic properties of the two synthetic eicosapeptides were checked, with RNA, after recombination with S-protein in various molar ratios. The Ile[8],Orn[10]-S-peptide forms a 16% active partially synthetic ribonuclease at a molar ratio of 100:1 with S-protein while the Ala[8],Orn[10]-S-peptide gives 20 and 28% activation of S-protein at molar ratios of 10:1 and 100:1, respectively. A tentative explanation of the different activating potencies of the two analogs is given.

Syntheses of four eicosapeptide analogs of the N-terminal amino acid sequence of bovine pancreatic ribonuclease A[2] have been carried out in our laboratory[3-6] in order to study the influence of amino acid replacements on the ability of S-peptide to bind S-protein and to regenerate ribonuclease activity.

We planned our substitutions on the assumption that conformational features may be very important for the correct association of S-peptide with S-protein.

Experiments of Vithayathil and Richards[7,8] by modifying either the three amino or the four carboxyl groups of S-peptide showed that the charge on S-peptide can be varied from $-3$ to $+4$ without destruction of its potential activity, and groups other than charged side chains must be significant in the catalytic properties.

However, Finn and Hofmann[9] and Hofmann, *et al.*,[10] by studying a series of partially synthetic RNase S′, showed that at least two of the four carboxyl groups of S-peptide, *i.e.*, Glu-2 and Asp-14, are significantly involved in binding.

Internal interactions involving methionine-13 were suggested by Vithayathil and Richards[11] and by Finn and Hofmann.[9] Since alkylation of such a residue led to a weaker binding, although enzymic activity was not greatly diminished at large excess of S-protein, the hydrophobic type of bond seems plausible. Richards[12] carried out difference spectra of S-peptide alone and in the presence of S-protein, and observed a significant change in the phenylalanine region of 255–270 mμ.

The striking similarity of this curve with the difference spectrum produced between Ac-Phe-OMe in water and in a cationic detergent solution[13] would seem to admit the conclusion of an altered environment of at least one phenylalanyl residue during the peptide–protein binding process, and it is likely that a change in the polar nature of such an environment is involved.[12]

These findings suggest that the polar or apolar character of some side-chain residues is strictly connected with the capacity of S-peptide to bind.

A possible model[14] of the structure of S-peptide, when it is bound to S-protein, was built bearing in mind the above-mentioned information and on the basis of conformational potential energy calculations carried out by Liquori, *et al.*,[15-17] as a function of the angles of rotation about the skeletal bonds of each residue (Figure 1).

While a definite conformation could not be assigned to residues 1–3 and 14–20, the section including residues 4–13 could be easily disposed in an α-helical conformation which clearly presents two sides.

(1) The peptides and peptide derivatives mentioned are of L configuration. For a simpler description the customary L designation for individual amino acid residues is omitted. The following abbreviations [IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966)] are used: Z = benzyloxycarbonyl, BOC = *t*-butyloxycarbonyl, Ac = acetyl, OMe = methyl ester, OEt = ethyl ester, OBu[t] = *t*-butyl ester, ON*p* = *p*-nitrophenyl ester, DMF = dimethylformamide, TFA = trifluoroacetic acid.

(2) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, 44, 162 (1958): RNase A, the principal chromatographic component of beef pancreatic ribonuclease; RNase S, subtilisin modified RNase A; S-peptide, the eicosapeptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein.

(3) E. Scoffone, F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, A. Scatturin, and A. Marzotto, *Tetrahedron Letters*, 943 (1966).

(4) E. Scoffone, R. Rocchi, F. Marchiori, A. Marzotto, A. Scatturin, A. M. Tamburro, and G. Vidali, *J. Chem. Soc., C*, 606 (1967).

(5) R. Rocchi, F. Marchiori, L. Moroder, A. Fontana, and E. Scoffone, *Gazz. Chim. Ital.*, **96**, 1537 (1966).

(6) F. Marchiori, R. Rocchi, L. Moroder, and E. Scoffone, *ibid.*, **96**, 1549 (1966).

(7) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 1029 (1960).

(8) P. J. Vithayathil and F. M. Richards, *ibid.*, **236**, 1380 (1961).

(9) F. M. Finn and K. Hofmann, *J. Am. Chem. Soc.*, **87**, 645 (1965).

(10) K. Hofmann, F. M. Finn, M. Limetti, J. Montibelle, and G. Zanetti, *ibid.*, **88**, 3633 (1966).

(11) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 2343 (1960).

(12) F. M. Richards and A. D. Logue, *ibid.*, **237**, 3693 (1962).

(13) S. Yanari and F. A. Bovey, *ibid.*, **235**, 2818 (1960).

(14) See the thesis by R. Filippi carried out in our laboratory and submitted in fulfillment of the requirements of the doctorate of the University of Padova (Feb 1966).

(15) A. M. Liquori, "Chimica delle Macromolecole," Consiglio Nazionale delle Ricerche, Rome, 1963, p 209.

(16) P. De Santis, E. Giglio, A. M. Liquori, and A. Ripamonti, *J. Polymer Sci.*, 1, 1383 (1963).

(17) P. De Santis, E. Giglio, A. M. Liquori, and A. Ripamonti, *Nature*, **206**, 4983 (1965).

**Chart I.** Amino Acid Sequence of S-Peptide and Its Synthetic Analogs

S-peptide

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-O

$Tyr^{8},Orn^{10}$-S-peptide[6]

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Tyr-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH

$Ile^{8},Orn^{10}$-S-peptide

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Ile-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH

$Ala^{8},Orn^{10}$-S-peptide

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Ala-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH

The first one containing the side chains of residues 6, 7, 9, and 10 is mainly hydrophilic and the second one, with the residues 4, 5, 8, 11, 12, and perhaps the methionine in position 13, is clearly hydrophobic.

If this model is correct one may assume that the hydrophobic face should be involved in the binding of S-peptide to S-protein while the hydrophilic one should be directed toward the exterior of the molecule, in contact with the solvent.

This idea fully agrees with the chemical evidence about the mutual position of the hystidyl residues in positions 12 and 119 and with the above-mentioned behavior of phenylalanine-8 during the binding process.

The helical model of S-peptide was clearly speculative when we began to plan our syntheses, but in 1966, Prothero[18] published a paper dealing with the correlation between the distribution of amino acids and $\alpha$-helices and, for ribonuclease, one of the predicted helical segments was near the N-terminal end including residues 2–11.

Moreover, early in 1967 a model of the polypeptide chain of bovine pancreatic ribonuclease based on a 2-A electron density map was proposed by Harker's group.[19] Although at the scale to which the map was drawn it was not easy to recognize all the side groups, many features of the molecule were visible.

The amino end sticks out quite independently of the rest of the molecule, and the only obvious helical segment is about two turns in the region of 5–12 near the amino end, even though there are other regions where there is a suggestion of helical conformation.

The consequence of this hypothesis is that any substitution of amino acids, not directly connected with the active site of the molecule, which neither changes the polarity of the side-chain residues nor has a helix-destabilizing effect is not expected to affect drastically the capacity of S-peptide to bind.

The results of our substitution at arginine-10[3–5] and the effect of the chemical modification previously carried out on S-peptide[7–11] fully agree with this working hypothesis.

A possible explanation of the fact that $Orn^{10}$-S-peptide[4] and des(15–20)-S-peptide[9] still possess potential activity when the glutamine-11 has been substituted by the glutamyl residue can be given by admitting that the side chain of the 11th residue lies in the border line region between the hydrophilic face and the hydrophobic one.

In order to assess the binding contribution of the residue in position 8, we carried out the synthesis of $Tyr^{8},Orn^{10}$-S-peptide (Chart I) and demonstrated[6] that substitution of both the phenylalanyl (position 8) and the arginyl (position 10) residues by tyrosyl and ornithyl residues, respectively, has virtually no effect on the ability of the resulting modified S-peptide to restore ribonuclease activity, after recombination with S-protein in a 1:1 molar ratio.

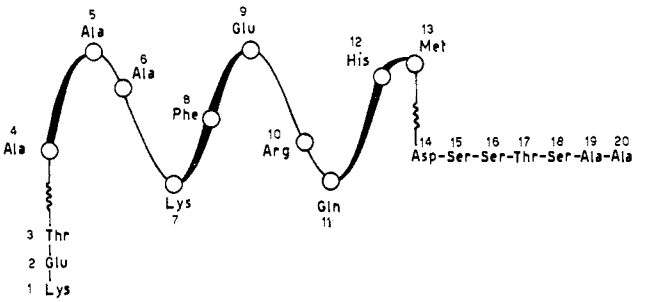



Figure 1. S-Peptide model where residues 4–13 are disposed in $\alpha$-helical conformation.

Moreover we found that, in $Tyr^{8},Orn^{10}$-RNase S′,[6] the tyrosyl residue in position 8 has the characteristic anomalous behavior of the three "buried" tyrosines of RNase A and S with regard to ionization[20] and spectral properties (unpublished results).

These findings are consistent with the hypothesis of an apolar environment for the "new" tyrosyl residue and agree with the Richards' conclusion of an altered environment of at least one phenylalanyl residue during the peptide–protein binding process.

It was interesting to see whether the side-chain aromatic character of the amino acid residue in position 8 is a fundamental requisite for correct peptide–protein association, or if an aliphatic, hydrophobic group can substitute the phenyl ring without marked influence on such an association.

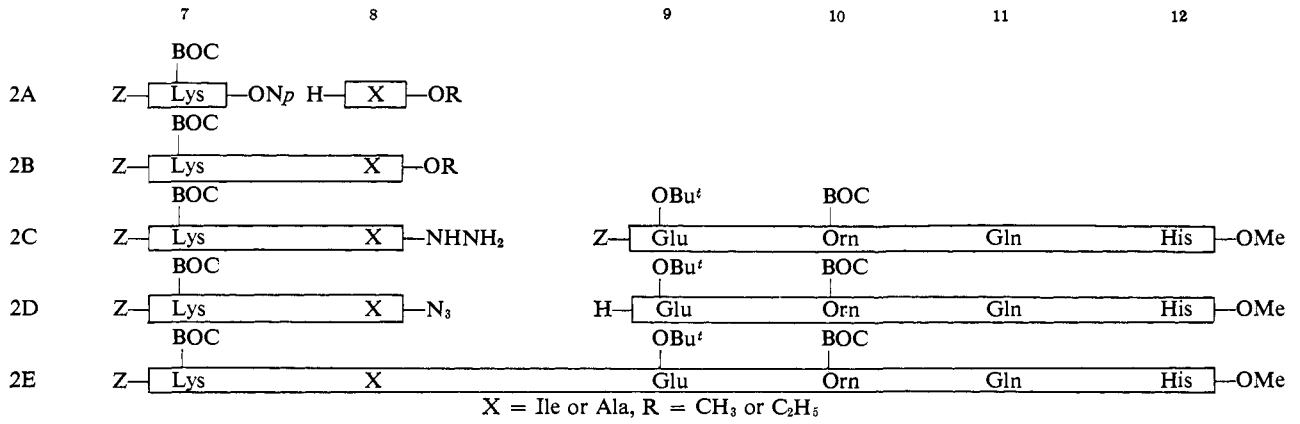
Moreover in order to explore the influence of the molecular dimensions of such an aliphatic group on the peptide–protein binding process, we synthetized both $Ile^{8},Orn^{10}$- and $Ala^{8},Orn^{10}$-S-peptide (Chart I). The ability of the synthetic materials to generate ribonuclease activity with S-protein in varying ratios was tested with RNA as substrate. The activities of the two partially synthetic RNase analogs expressed as a percentage of RNase S′ activity are listed in Table I.

(18) J. W. Prothero, *Biophys. J.*, 6, 367 (1966).

(19) G. Kartha, J. Bello, and D. Harker, *Nature*, 213, 862 (1967).

(20) E. Scoffone, F. Marchiori, A. Marzotto, and R. Rocchi, Proceedings of the VIIIth European Peptide Symposium, Noordwijk, Holland, Sept 1966, H. C. Beyerman, A. Van de Linde, and W. Maassen Van Den Brink, Ed., North-Holland Publishing Co., Amsterdam, 1967, p 280.

**Chart II.** Synthesis of the 7-12 Sequences




In order to reduce the methionine sulfoxide, eventually present, to methionine, the synthetic eicosapeptides were dissolved in 1% aqueous thioglycolic acid and incubated under nitrogen at 45° according to the procedure described by Hofmann, *et al.*[10]

Ribonuclease assays were performed with the reduced eicosapeptides as well as with the untreated materials, and no significant differences were found in the ability of such materials to activate S-protein.

**Table I.** S-Protein Activating Capacity of S-Peptide Analogs. Substrate RNA

| S-peptide analogs | Molar ratio of protein-peptide | RNase S′ activity, % |
|---|---|---|
| Tyr$^8$,Orn$^{10}$ [a] | 1:1 | 82 |
| | 1:10 | 82 |
| | 1:100 | 82 |
| Ile$^8$,Orn$^{10}$ | 1:1 | 0 |
| | 1:10 | 0 |
| | 1:100 | 16 |
| Ala$^8$,Orn$^{10}$ | 1:1 | 0 |
| | 1:10 | 20 |
| | 1:100 | 28 |

[a] See ref 6 and 20.

Replacement of the aromatic side chain of the phenylalanyl residue with the branched aliphatic chain of isoleucine is accompanied by a marked decrease in the ability of the synthetic material to restore ribonuclease activity.

A 16% activation of S-protein is obtained only at molar ratios of 100:1 in contrast to Tyr$^8$,Orn$^{10}$-S-peptide which activates 82% at molar ratios of 1:1.[20]

This observation suggests that the aromatic character of the phenyl ring of the phenylalanyl or tyrosyl residue is an important structural element in the binding with S-protein and, probably, a $\pi$-bonding interaction with some other aromatic residue in the protein fragment is involved.

The significant role played by the aromatic side-chain interactions in maintaining, for example, the structural integrity of myoglobin,[21] and in inducing and stabilizing the polymer formation of bovine pancreatic carboxypeptidase A,[22] has already been pointed out.

As the activating potency of both the Ala$^8$,Orn$^{10}$ and Ile$^8$,Orn$^{10}$ analogs is very low in comparison with the Tyr$^8$,Orn$^{10}$-S-peptide, it is reasonable to presume that the possible helix-destabilizing effect of the isoleucyl residue, in contrast to the alanyl residue, is not important.

A possible explanation of the finding that the Ala$^8$,Orn$^{10}$-S-peptide forms a 20% active partially synthetic ribonuclease with S-protein at a molar ratio about ten times lower than the Ile$^8$,Orn$^{10}$-S-peptide is that in the alanine-substituted peptide only the aromatic character of the "binding site" is destroyed while, by introducing the isoleucyl residue, this effect can be accompanied by a steric hindrance which further lowers the peptide's ability to bind.

## Peptide Syntheses

The synthetic route to Ile$^8$,Orn$^{10}$- and Ala$^8$,Orn$^{10}$-S-peptide, illustrated in Charts II and III, is similar to that we used for the preparation of other analogs.[3-6]

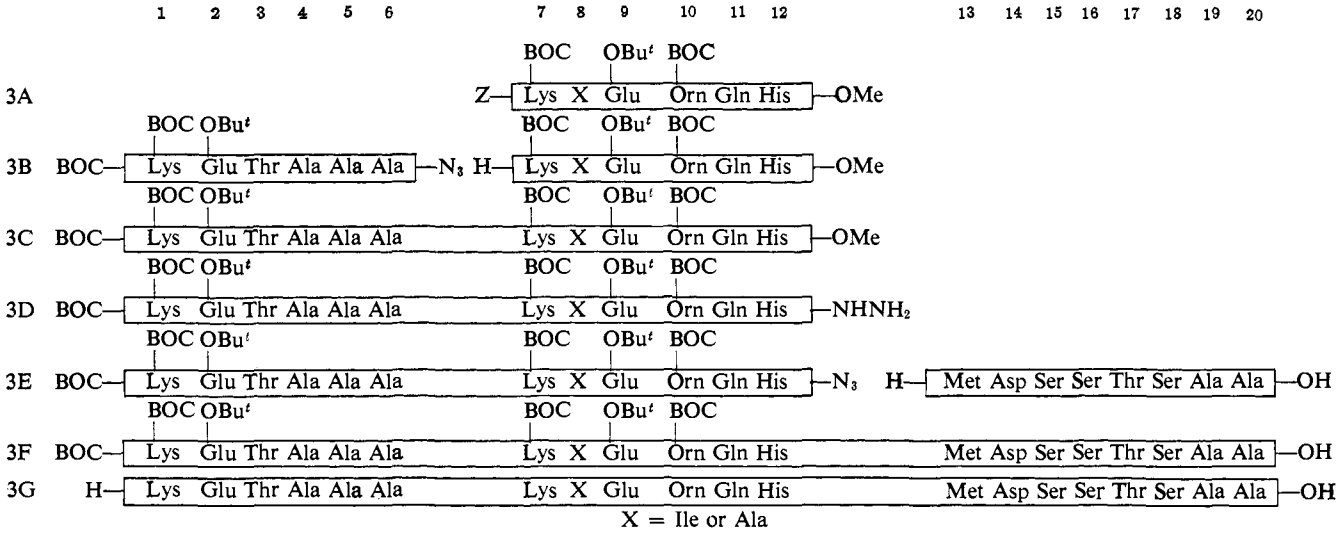
The dipeptides N$^\alpha$-benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllylsylisoleucine methyl ester (2B, Ile$^8$ 7–8) and N$^\alpha$-benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysylalanine ethyl ester (2B, Ala$^8$ 7–8) were transformed into the corresponding hydrazides (2C, Ile$^8$ 7–8 and 2C, Ala$^8$ 7–8) and condensed, by an azide coupling step, with the tetrapeptide $\gamma$-*t*-butylglutamyl-N$^\delta$-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester[23] (2D, 9–12).

The resulting hexapeptides 2E (or 3A), Ile$^8$ 7–12 and 2E (or 3A), Ala$^8$ 7–12 were hydrogenated and coupled with N$^\alpha$,N$^\epsilon$-di-*t*-butyloxycarbonyllysyl-$\gamma$-*t*-butylglutamylthreonylalanylalanylalanine azide[24] (3B, 1–6) to give the protected dodecapeptide esters (3C, Ile$^8$ 1–12 and 3C, Ala$^8$ 1–12) which were converted into hydrazides (3D, Ile$^8$ 1–12 and 3D, Ala$^8$ 1–12) in the usual manner. The corresponding azides (3E, Ile$^8$ 1–12 and 3E, Ala$^8$ 1–12) were then treated with the octapeptide methionylaspartylserylserylthreonylserylalanylalanine[25] (3E, 13–20) to give the partially protected eicosapeptides 3F, Ile$^8$ 1–20 and 3F, Ala$^8$ 1–20. The protecting groups were removed by exposure to TFA, and the crude eicosapeptides, 3G, Ile$^8$ 1–20 and 3G, Ala$^8$ 1–20, were purified by chromatography through an Amberlite CG 50 column eluted with 0.2 *M* sodium phosphate, desalted by gel filtration on Sephadex G 25 using 5% acetic acid as an eluent, and lyophilized.

The chemical homogeneity of both the S-peptide analogs was evaluated by electrophoresis at different

(21) J. R. Cann, *Biochemistry*, 4, 2368 (1965).
(22) J. L. Bethune, *ibid.*, 4, 2698 (1965).
(23) F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, and E. Scoffone, *J. Chem. Soc., C*, 81 (1967).
(24) R. Rocchi, F. Marchiori, A. Scatturin, and E. Scoffone, *ibid.*, 86 (1967).
(25) F. Marchiori, R. Rocchi, L. Moroder, G. Vidali, and E. Scoffone, *ibid.*, 89 (1967).

**Chart III.** Synthesis of the 1–20 Sequences




pH values and quantitative amino acid analysis of the acid hydrolisates.

Aminopeptidase M (AP-M) was employed for evaluation of the stereochemical homogeneity of synthetic materials according to the procedure described by Hofmann, *et al.*[10]

## Experimental Section[26]

RNase A was prepared from bovine pancreatic ribonuclease (Fluka AG four times crystallized) by the procedure of Crestfield, *et al.*[27] RNase S, S-protein, and S-peptide were prepared from RNase A, essentially by the method of Richards.[28-30] Aminopeptidase M (AP-M) was obtained from Rohm and Haas GmbH, Darmstadt, West Germany.

The crude S-peptide analogs (100–200 mg), obtained by treatment of the partially protected eicosapeptides with TFA, were dissolved in 0.2 *M* sodium phosphate buffer (pH 6.47) and purified by chromatography through an Amberlite CG 50 column (1.8 × 90 cm) with the same phosphate buffer as the eluent.

Individual fractions (2.5 ml) were collected (rate *ca.* 15 ml/hr) and the products detected by the ninhydrin test and the Pauly reaction. The ninhydrin, Pauly-positive fractions were pooled, concentrated under reduced pressure, lyophilized, and desalted by passing through a Sephadex G 25 column (1.8 × 140 cm) with 5% acetic acid as the eluent. The peptide was detected as described above and the peptide-containing fractions were pooled, concentrated to a syrup, and lyophilized from water to constant weight. Commercial yeast RNA (sodium salt) was obtained from BDH Laboratory Chemical Division and purified by exhaustive dialysis, first against 0.1 *M* sodium chloride and then against water.[31]

**N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysylisoleucine Methyl Ester (2B, Ile$^8$ 7–8).** N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[23,32] (3.09 g, 6.0 mmoles) was added to a solution of isoleucine methyl ester hydrochloride[33] (1.14 g, 6.3 mmoles) in pyridine (80 ml) containing triethylamine (1.8 ml).

The reaction mixture was kept 24 hr at room temperature and the solvent removed under reduced pressure. The residue, taken up in ethyl acetate, was washed with 5% sodium carbonate, 5% citric acid, and water. The solution was dried over sodium sulfate and concentrated to a small volume *in vacuo*. The compound was precipitated by addition of petroleum ether (bp 30–60°); yield 2.74 g (90%), mp 40–42°, $[\alpha]^{22}D$ −15 ± 0.2° (*c* 1.0, methanol), $R_{f_1}$ 0.90, $R_{f_2}$ 0.98, single ninhydrin-negative, chlorine-positive spot.

*Anal.* Calcd for $C_{26}H_{41}N_3O_7$ (507.61): C, 61.5; H, 8.1; N, 8.3. Found: C, 61.7; H, 8.3; N, 8.3.

**N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysylalanine ethyl ester (2B, Ala$^8$ 7–8)** was prepared from N$^\alpha$-benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[23,32] (3.09 g, 6.0 mmoles) and alanine ethyl ester hydrochloride[33] (0.97 g, 6.3 mmoles) by the above reported procedure used for the preparation of 2B, Ile$^8$ 7–8; yield 2.59 g (90%), mp 98–99°, $[\alpha]^{22}D$ −24.2 ± 0.2° (*c* 1.0, ethanol), $R_{f_1}$ 0.85, $R_{f_2}$ 0.97, single ninhydrin-negative, chlorine-positive spot.

*Anal.* Calcd for $C_{24}H_{37}N_3O_7$ (479.56): C, 60.1; H, 7.8; N, 8.8. Found: C, 60.0; H, 7.9; N, 8.8.

**N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysylisoleucine Hydrazide (2C, Ile$^8$ 7–8).** N$^\alpha$-Benzyloxycarbonyl-N$_\epsilon$-*t*-butyloxycarbonyllysylisoleucine methyl ester (2.54 g, 5.0 mmoles) was dissolved in methanol (12 ml), and hydrazine hydrate (5 ml) was added. After 5 days at room temperature the precipitated product was collected, washed with methanol and ether, and dried *in vacuo* over phosphorus pentoxide and concentrated sulfuric acid, yielding 2.0 g (79%), mp 161–162°, $[\alpha]^{22}D$ −32.7 ± 0.2° (*c* 1.0 glacial acetic acid), $R_{f_1}$ 0.85, $R_{f_2}$ 0.92, single chlorine and picryl chloride-positive spot.

*Anal.* Calcd for $C_{25}H_{41}N_5O_6$ (507.62): C, 59.1; H, 8.1; N, 13.8. Found: C, 59.2; H, 8.1; N, 13.7.

**N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxycarbonyllysylalanine Hydrazide (2C, Ala$^8$ 7–8).** N$^\alpha$-Benzyloxycarbonyl-N$^\epsilon$-*t*-butyloxy-

(26) The melting points were determined by the Tottoli's capillary melting point apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. The acid hydrolyses were carried out with 6 *N* hydrochloric acid, in sealed evacuated ampoules, for 22 hr at 110°. Aminopeptidase M digests were prepared in the manner described by Hofmann, *et al.*[10] The amino acid composition of acid and enzymic hydrolysates was determined by quantitative analysis using a Technicon amino acid analyzer. Ascending thin layer chromatography was performed on Silica Gel G (Merck) with the following solvent systems: $R_{f_1}$, 1-butanol–glacial acetic acid–water (3:1:1); $R_{f_2}$, ethyl acetate–pyridine–glacial acetic acid–water (60:20:6:14). Electrophoreses were carried out on Whatman No. 1 filter paper, at a gradient of about 20 v/cm, for 3 hr, at pH 1.9 (25% acetic acid), 3.5 and 6.4 [pyridine–acetic acid–water (1:10:89 and 5:0.2:95)], and 9.4 ($Na_2CO_3$–$NaHCO_3$): G. E. Delory and E. J. King, *Biochem. J.*, **39**, 245, (1945). The chlorine [H. N. Rydon and P. Smith, *Nature*, **196**, 922 (1952)] and the Pauly (K. Randerath, "Thin Layer Chromatography," Academic Press Inc., New York, N. Y., 1963, p 176) tests were carried out according to the literature.

The hydrazides were also revealed by spraying the chromatograms with a 1% picryl chloride solution in 95% ethanol, followed by exposure to ammonia vapors. Catalytic hydrogenolysis were carried out in the indicated solvent, containing a few drops of glacial acetic acid, over 10% palladized charcoal, for the indicated time. Unless stated otherwise, solvents were evaporated at a bath temperature of 40–50° in a rotatory evaporator. The enzymic activity of the partially synthetic, modified ribonucleases was determined, with RNA substrate, essentially as described by M. Kunitz, *J. Biol. Chem.*, **164**, 563 (1946).

(27) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 618 (1963).

(28) F. M. Richards and P. J. Vithayathil, *ibid.*, **234**, 1459 (1959).

(29) G. Gordillo, P. J. Vithayathil, and F. M. Richards, *Yale J. Biol. Med.*, 34, 582 (1962).

(30) A. Marzotto, A. Scatturin, G. Vidali, and E. Scoffone, *Gazz. Chim. Ital.*, **94**, 760 (1964).

(31) D. Wellner, H. J. Silman, and M. Sela, *J. Biol. Chem.*, **238**, 1324 (1963).

(32) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).

(33) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud, and E. Sandrin, *ibid.*, **38**, 1941 (1955).

carbonyllysylalanine ethyl ester (2.4 g, 5.0 mmoles) was dissolved in ethanol (12 ml), and hydrazine hydrate (5 ml) was added. After 6 days at room temperature the solvent was removed up to one-third of the initial volume and precipitation occurred by addition of ether. The compound was collected, washed with ethanol–ether (1:1 by volume) and ether, and dried *in vacuo* over phosphorus pentoxide and concentrated sulfuric acid, yielding 1.7 g (73%), mp 132–133°, $[\alpha]^{22}D$ −27.5 ± 0.2° (*c* 1.0, glacial acetic acid), $R_{f_1}$ 0.75, $R_{f_2}$ 0.92, single chlorine and picryl chloride-positive spot.

*Anal.* Calcd for $C_{22}H_{35}N_5O_6$ (465.54): C,5 6.7; H, 7.6; N, 15.0. Found: C, 56.6; H, 7.6; N, 14.9.

**$N^{\alpha}$-Benzyloxycarbonyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylisoleucyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester [2E (or 3A), Ile$^8$ 7–12].** Sodium nitrite (1 *M*, 2.7 ml) was added to a solution of 2C, Ile$^8$ 7–8 (1.015 g, 2.0 mmoles) in a mixture of glacial acetic acid (15 ml), 1 *N* hydrochloric acid (4 ml), and saturated sodium chloride (5 ml) cooled to −10°. After stirring at −10° for 15 min, precooled, saturated sodium chloride (30 ml) and ice-cold water (10 ml) were added, and the $N^{\alpha}$-benzyloxycarbonyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylisoleucine azide (2D, Ile$^8$ 7–8) was extracted with three 20 ml-portions of ethyl acetate cooled to −10°.

The combined organic layers were quickly washed with ice-cold, saturated solutions of potassium carbonate and sodium chloride and dried for 10 min at −10° over sodium sulfate.

Simultaneously benzyloxycarbonyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester[23] (2C, 9–12) (1.69 g, 2.0 mmoles) was dissolved in methanol and hydrogenated 2 hr and the catalyst filtered off. The filtrate was evaporated to dryness under reduced pressure, and the residue ($R_{f_1}$ 0.45, single ninhydrin-, chlorine-, and Pauly-positive spot) was dissolved in DMF (20 ml) containing triethylamine (0.6 ml) and added to the ethyl acetate solution of the protected dipeptide azide (2D, Ile$^8$ 7–8) prepared above.

The resulting solution was concentrated under reduced pressure, at 0° to remove most of the ethyl acetate and then allowed to react at 5° for 5 days, whereupon ether was added.

The precipitate was collected, washed with ether, and crystallized twice from methanol–water; yield 1.4 g (60%), mp 224–225°, $[\alpha]^{22}D$ −15.2 ± 0.3° (*c* 1.0, DMF), $R_{f_1}$ 0.65, $R_{f_2}$ 0.70, single ninhydrin-negative, chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{1.05}$, $Orn_{1.00}$, $Ile_{0.98}$, $Glu_{2.10}$, $His_{0.98}$.

*Anal.* Calcd for $C_{56}H_{89}N_{11}O_{16}$ (1172.42): C, 57.4; H, 7.6; N, 13.1. Found: C, 57.0; H, 7.5; N, 13.1.

**$N^{\alpha}$-Benzyloxycarbonyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylalanyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester [2E (or 3A), Ala$^8$ 7–12].** The condensation of 2C, Ala$^8$ 7–8 (0.93 g, 2.0 mmoles) with 2D, 9–12, obtained by hydrogenolysis in methanol from 2C, 9–12 (1.69 g, 2.0 mmoles), was carried out by the azide procedure, as described above for 2E, Ile$^8$ 7–12, yielding 1.04 g (46%), mp 205–207°, $[\alpha]^{22}D$ −14.3 ± 0.3° (*c* 1.0, DMF), $R_{f_1}$ 0.55, $R_{f_2}$ 0.62, single ninhydrin-negative, chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{1.00}$, $Orn_{1.00}$, $Ala_{0.97}$, $Glu_{2.05}$, $His_{0.95}$.

*Anal.* Calcd for $C_{53}H_{83}N_{11}O_{16}$ (1130.34): C, 56.3; H, 7.4; N, 13.6. Found C, 55.9, H, 7.3; N, 13.8.

**$N^{\alpha}$,$N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylisoleucyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, Ile$^8$ 1–12).** The protected hexapeptide ester (3A, Ile$^8$ 7–12) (0.415 g, 0.354 mmole) was dissolved in DMF (20 ml) and hydrogenated for 3 hr when active charcoal was added and the catalyst removed by centrifugation.

$N^{\alpha}$,$N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanine azide[24] (3B, 1–6) (0.31 g, 0.354 mmole) and triethylamine (0.13 ml) were added, and the reaction mixture was stirred at 5° for 3 days after which a second portion (0.08 g) of the above azide (3B, 1–6) was added. After stirring for 5 days more at 5°, the solution was filtered, and water (100 ml) was added to the filtrate.

The resulting precipitate was collected by centrifugation, washed with water and ether, and crystallized twice from DMF–ether yielding 0.409 g (62%), mp 250–253° dec, $[\alpha]^{22}D$ −21.4 ± 0.5° (*c* 0.5, DMF), $R_{f_1}$ 0.70, $R_{f_2}$ 0.80, single ninhydrin-negative, chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{2.05}$, $Orn_{1.05}$, $Glu_{3.00}$, $Thr_{0.97}$, $Ala_{2.95}$, $Ile_{0.95}$, $His_{0.97}$.

*Anal.* Calcd for $C_{86}H_{148}N_{18}O_{27}$ (1866.31): C, 55.3; H, 8.0; N, 13.5. Found: C, 54.7; H, 8.3; N, 13.2.

**$N^{\alpha}$,$N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylalanyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, Ala$^8$ 1–12).** The condensation of 3B, 1–6[24] (0.35 g, 0.4 mmole, and 0.088 g, 0.1 mmole, as second portion), with 3B, Ala$^8$ 7–12, obtained by hydrogenolysis in DMF from 3A, Ala$^8$ 7–12 (0.452 g, 0.4 mmole), was carried out as described above for 3C, Ile$^8$ 1–12, yielding 0.408 g (56%), mp 230–233° dec, $[\alpha]^{22}D$ −14.7 ± 0.5° (*c* 1.0, DMF), $R_{f_1}$ 0.70, $R_{f_2}$ 0.80, single ninhydrin-negative, chlorine- and Pauly- positive spot; amino acid ratios in acid hydrolysate: $Lys_{2.00}$, $Orn_{1.05}$, $Glu_{2.95}$, $Thr_{0.95}$, $Ala_{3.95}$, $His_{0.95}$.

*Anal.* Calcd for $C_{83}H_{142}N_{18}O_{27}$ (1824.23): C, 54.6; H, 7.8; N, 13.8. Found: C, 54.0; H, 7.6; N, 13.6.

**$N^{\alpha}$,$N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylisoleucyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, Ile$^8$ 1–12).** The dodecapeptide methyl ester (3C, Ile$^8$ 1–12) (0.854 g, 0.457 mmole) was dissolved in DMF (30 ml), and hydrazine hydrate (0.9 ml) was added.

The solution was heated for 1 hr at 70°, hydrazine hydrate (0.45 ml) was then added, and the reaction mixture was kept overnight at 50° and then 3 days more at room temperature.

The addition of water gave a precipitate, which was collected by centrifugation, washed with water (three times) and ether, and dried *in vacuo*, first over concentrated sulfuric acid and then at 110°. The product (0.75 g, 88%) had mp 255–257° dec, $[\alpha]^{22}D$ −29.8 ± 0.5° (*c* 1.0, glacial acetic acid), $R_{f_1}$ 0.75, $R_{f_2}$ 0.80, single chlorine-, picryl chloride-, and Pauly-positive spot.

*Anal.* Calcd for $C_{85}H_{148}N_{20}O_{26}$ (1866.32): C, 54.7; H,8 .0; N, 15.0. Found: C, 53.7; H, 7.8; N, 14.8.

**$N^{\alpha}$,$N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-$N^{\epsilon}$-*t*-butyloxycarbonyllysylalanyl-γ-*t*-butylglutamyl-$N^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, Ala$^8$ 1–12).** This compound was obtained from 3C, Ala$^8$ 1–12 (0.596 g, 0.327 mmole) by the same procedure reported above for the preparation of 3D, Ile$^8$ 1–12; yield 0.358 g (60%), mp 242–244° dec, $[\alpha]^{22}D$ −23.5 ± 0.5° (*c* 1.0, glacial acetic acid), $R_{f_1}$ 0.70, $R_{f_2}$ 0.75, single chlorine-, picryl chloride-, and Pauly-positive spot.

*Anal.* Calcd for $C_{82}H_{142}N_{20}O_{26}$ (1824.24): C, 54.0; H, 7.8; N, 15.4. Found: C, 53.9; H, 7.8; N, 15.3.

**Lysylglutamylthreonylalanylalanylalanyllysylisoleucylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, Ile$^8$ 1–20).** Sodium nitrite (1 *M*, 0.5 ml) was added to a solution of 3D, Ile$^8$ 1–12 (0.265 g, 0.142 mmole) in a mixture of glacial acetic acid (10 ml), 1 *N* hydrochloric acid (1 ml), and 20% sodium chloride (2 ml) at −10°.

After stirring for 15 min at −10°, precooled 20% sodium chloride (100 ml) was added, and the resulting precipitate was collected and washed with ice-cold water. The still wet material was dissolved in DMF (20 ml) at −10° and dried over sodium sulfate. The drying agent was filtered off and methionylaspartylserylserylthreonylserylalanylalanine[25] (3E, 13–20) (0.25 g, 0.284 mmole, as monoacetate, trihydrate) and triethylamine (0.14 ml) were added. The reaction mixture was stirred for 7 days at 5° and for 1 day at room temperature, filtered, concentrated to 10 ml, and diluted with water (150 ml). The resultant precipitate was centrifuged, washed with water and ether, and dried over phosphorus pentoxide yielding 0.26 g (70%).

The crude material (3F, Ile$^8$ 1–20) was dissolved in anhydrous TFA (2 ml), and the solution was kept for 150 min at room temperature.

Ice-cold ether (50 ml) was added, and after 30 min at −10° the peptide was collected by centrifugation, washed with ether, and dried.

The residue, dissolved in 0.2 *M* sodium phosphate buffer, was purified by passing through an Amberlite CG 50 column, desalted by gel filtration on a Sephadex G 25 column, and lyophilized as described previously.

The product (3G, Ile$^8$ 1–20) (0.096 g, 46%) had $[\alpha]^{22}D$ −72 ± 2° (*c* 0.1, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{2.14}$, $Orn_{1.10}$, $Glu_{2.80}$, $Thr_{1.90}$, $Ala_{4.85}$, $Ile_{1.00}$, $His_{1.00}$, $Met_{0.90}$, $Asp_{1.10}$, $Ser_{2.80}$; amino acid ratios in AP-M digest: $Lys_{2.10}$, $Orn_{1.05}$, $Glu_{1.98}$, $Thr_{1.99}$, $Ala_{4.87}$, $Ile_{1.00}$, $Gln_{0.94}$, $His_{1.00}$, $Met_{0.89}$, $Asp_{1.00}$, $Ser_{2.85}$.

The enzymic activities, after recombination with S-protein in different ratios, are reported in Table I.

**Lysylglutamylthreonylalanylalanylalanyllysylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, Ala$^8$ 1–20).** The condensation of 3D, Ala$^8$ 1–12 (0.26 g, 0.142 mmole) with 3E, 13–20[25] (0.25 g, 0.284 mmole as

monoacetate, trihydrate) by the azide procedure was carried out as described above for 3G, Ile[8] 1–20 and gave the partially protected Ala[8],Orn[10]-S-peptide (3F, Ala[8] 1–20, 0.18 g, 50%).

Treatment with anhydrous TFA, purification on Amberlite CG 50, and on Sephadex G 25, followed by lyophilization, gave the pure Ala[8], Orn[10]-S-peptide (3G, Ala[8] 1–20) (0.029 g, 20%), $[\alpha]^{22}D$ $-79.5 \pm 2°$ ($c$ 0.1, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{2.10}$, $Glu_{2.90}$, $Thr_{1.85}$, $Ala_{5.80}$,$Orn_{1.10}$, $His_{1.00}$, $Met_{0.96}$, $Asp_{1.07}$, $Ser_{2.85}$; amino acid ratios in AP-M digest: $Lys_{2.05}$, $Glu_{2.05}$, $Thr_{1.90}$, $Ala_{5.85}$, $Orn_{1.05}$, $Gln_{1.00}$, $His_{1.00}$, $Met_{0.95}$, $Asp_{1.10}$, $Ser_{2.80}$. The enzymic activity data are listed in Table I.

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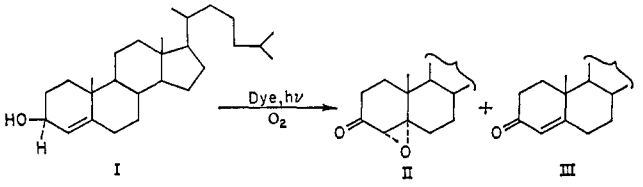
# Communications to the Editor

## Evidence for the Participation of $^1\Sigma_g^+$ and $^1\Delta_g$ Oxygen in Dye-Sensitized Photooxygenation Reactions. I[1]

*Sir:*

The dye-sensitized photooxygenation of cholest-4-en-3β-ol (I) has been studied in considerable detail,[2] but until now the mechanism of this oxidation reaction has not successfully been incorporated into discussions of the "singlet oxygen" mechanism.[3] Sensitized photooxygenation of this compound yields only two products, an epoxy ketone (II) and an enone (III). However, in




I II III

contrast to other sensitized photooxygenations which are believed to involve singlet oxygen molecules (presumably $^1\Delta_g$),[4-8] this reaction gives an enone:epoxy ketone (III:II) product distribution ratio which varies from 30:1 to 1:5, depending upon the nature of the sensitizer used. To account for these observations, we propose *that both $^1\Sigma_g^+$ and $^1\Delta_g$ oxygen molecules are involved as reaction intermediates, that these two species exhibit different chemical reactivities, and that the observed variation in product distribution with sensitizer results from a variation in the relative amounts of $^1\Sigma_g^+$ and $^1\Delta_g$ generated.* This suggestion is prompted by the following considerations.

We recently showed, on theoretical grounds, that quenching of excited triplet-state molecules by molecular oxygen occurs primarily by transfer of electronic excitation energy to the molecular oxygen.[9] With high-energy triplet-state sensitizers ($E_t > 50$ kcal) our calculations indicated that in the quenching process about 10 $^1\Sigma_g^+$ molecules are generated for every $^1\Delta_g$. With low-energy sensitizers ($E_t < 38$ kcal) energy conservation permits only $^1\Delta_g$ oxygen to be generated. Energy transfer from sensitizers with intermediate triplet-state energies ($50 > E_t > 38$ kcal) was not adequately treated by our theory and, in order to predict the $^1\Sigma_g^+:{}^1\Delta_g$ ratio expected from these sensitizers, we make use of experimental observations on triplet–triplet energy transfer.[10] Porter and Wilkinson found that, as the triplet-state energy of the sensitizer approaches (*e.g.*, within 5 kcal) the triplet state of the acceptor, the transfer probability is significantly reduced.[11] Assuming that similar results are obtained when oxygen is the acceptor, we expect the $^1\Sigma_g^+:{}^1\Delta_g$ ratio to smoothly decrease to zero as $E_t$ approaches 37.7 kcal, the energy of the $^1\Sigma_g^+$ state. These considerations allow us to construct the curve given in Figure 1a, showing the expected variation in the $^1\Sigma_g^+:{}^1\Delta_g$ ratio for a wide range of sensitizer triplet-state energies. If $^1\Sigma_g^+$ and $^1\Delta_g$ oxygen were to exhibit different chemical reactivities, this curve suggests that the product distribution would vary with triplet-state energy of the sensitizer in a predictable way.

To test for the possible involvement of $^1\Sigma_g^+$ and $^1\Delta_g$ oxygen as intermediates, we have measured the triplet-state energies of a number of the sensitizers which were used in the sensitized photooxygenation of I.[12] In Figure 1b we have plotted the enone:epoxy ketone (III:II) ratio obtained from the sensitized photooxygenation of I as a function of the triplet-state energy of the sensitizers.[2] It is apparent from a comparison of the curves in Figures 1a and 1b that the variation in product distribution with $E_t$ is well accounted for in terms of the predicted variation of the $^1\Sigma_g^+:{}^1\Delta_g$ ratio and the proposed involvement of both $^1\Sigma_g^+$ and $^1\Delta_g$ molecular oxygen as reaction intermediates. Low-energy sensitizers, which produce only $^1\Delta_g$, give a III:II ratio of

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