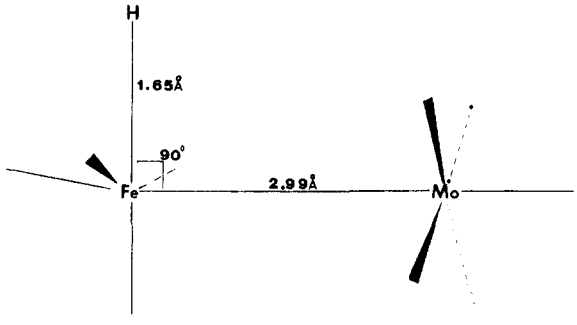
ployed in this study were similar to those used in our previous work.[8,9] The carbonyl and metal core were represented by a minimal basis set. The H and valence orbitals of the metals were represented by a double-ζ basis set. Although this is a small basis, the double-ζ character of the metals and hydrogen should lead to reasonable structures. Except for the plotting, all calculations were carried out on the chemistry department VAX 11/780 computer. The integrals and the Hartree–Fock–Roothaan[10] SCF calculations were done with the ATMOL 3 systems of programs.[11] The program MOPLOT was used to plot the wavefunctions of the molecular orbitals.[12] The maps were drawn by the program CONTOUR on a Xerox 9700 laser printing system[13] on the Texas A&M University Amdahl computers.

The optimized coordinates gave a H–Fe–Mo bond angle of 90°, a H–Fe bond length of 1.65 Å, and C_s symmetry. The calculated H position is seen in the molecule depicted below. Although we



have not mapped out the entire energy surface for the H, we have examined a number of points near bridging, semibridging, and terminal sites. The terminal site appears to be a global minimum. The eigenvectors resulting from the ab initio calculation produced the orbital contour maps seen in Figure 1, which show the orbitals in the *YZ* plane containing the H, Fe, and Mo atoms and 3CO groups. Figure 1a contains a contour plot of the HOMO which contains the heteronuclear bimetallic interactions. This orbital is clearly the bonding combination between the $HFe(CO)_4^-$ and $Mo(CO)_5$ fragments. The orbital is mainly Fe in character, as would be expected for a formal $HFe(CO)_4^-$ fragment with a dative bond to a $Mo(CO)_5$ fragment. Figure 1b shows the H–Fe interaction where the electron density lies toward the hydrogen as expected for a transition-metal hydride. The chemistry of these ions supports this description.[1,14]

There is circumstantial evidence to support our prediction of a terminal hydride and of its location. The occurrence of a bridging hydride ligand is rare for a heteronuclear metal–metal bond; examples of bimetallic complexes where the hydride is the only bridging ligand are even rarer.[15] The X-ray crystal structure (non-hydrogen portion) showed no bridging carbonyls and the infrared spectra showed no bands in the bridging carbonyl region.[1] Also, it is well-known that terminal M–H distances involving first-row transition metals usually lie in the range 1.4–1.7 Å; furthermore, the parent fragment, $[HFe(CO)_4]^-$ has an Fe–H bond length of 1.57 Å.[2] Very recently, Darensbourg and co-workers[16] have synthesized $[(Ph_3PAu)Fe(CO)_4W(CO)_5]^-$, which has a Ph_3PAu bound terminally to the iron. The isolobal analogy[17,18] would suggest that this Ph_3PAu complex has a structure similar to $[HFe(CO)_4W(CO)_5]^-$. The IR band patterns and C^{13} resonances are comparable, and X-ray data show the Fe–W and W–CO bond distances (as well as the arrangement of atoms) to be nearly identical for the two compounds. All evidence points to a terminal Fe–H with the W–H coupling arising indirectly through the Fe–W bond or directly through the delocalization of the Fe–H bond onto W, as shown in Figure 1b.

With modern computer systems, significantly larger molecules could be handled. Thus, the approach of freezing the heavy atoms at the X-ray structure and locating the H through ab initio calculations may be competitive with neutron diffraction in terms of cost and effort. It has advantages over molecular mechanical methods, because it needs neither estimates of M–H bond lengths nor assumptions about H sites (terminal, edge bridging, or face capping).

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Conformational Considerations in the Design of a Glucagon Analogue with Increased Receptor Binding and Adenylate Cyclase Potencies

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Glucagon is a peptide hormone produced and processed into its biologically active form in the pancreas and in the gut, whose primary biological role is the stimulation of glucose release and production. The most important biological form of the hormone is a linear 29 amino acid peptide (H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH). Structure–function studies have demonstrated that the C-terminal region of glucagon is important for binding of the hormone to its plasma membrane receptor.[1-3] The X-ray structure of a crystalline form of the hormone formed under basic conditions has been determined[4] and shows that this region has an α-helical structure. Numerous studies indicate that this α-helical structure is present in solution under a variety of conditions for glucagon.[5] Analysis

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of the C-terminal helix indicates that it is amphiphilic in nature.[6]

Epand and Liepnieks[7] have prepared the semisynthetic analogue [δ-(5-nitro-2-pyrimidyl)ornithine^{17,18}]glucagon for the purpose of extending the α-helix. The compound was found to be equipotent to glucagon in the adenylate cyclase assay and to bind to its receptor with half the affinity of glucagon.[7] Musso et al.[8] have reported two synthetic glucagon analogues that incorporate a model amphiphilic helix in the C-terminus of glucagon. One compound was inactive, but another, [Leu^{19,23,27},Glu^{21},Ala^{26},Gln^{28}]glucagon exhibited weak receptor binding activity.[9]

In order to examine the effect of enhanced helical potential (as measured by the method of Chou and Fasman[10]) in the C terminal of glucagon on its adenylate cyclase and receptor binding activities, we have designed and synthesized a glucagon analogue with enhanced α-helical potential in the C-terminal region in a manner similar to that suggested by Chou and Fasman.[11] Since little is known about the importance of the individual residues in this portion of glucagon structure for biological or binding potency,[2] we were as conservative as possible in structural modification. We report here the design and synthesis of [Lys^{17,18},Glu^{21}]glucagon, a glucagon analogue and much higher potency than the natural hormone in plasma membrane adenylate cyclase and receptor binding activities.

[Lys^{17,18},Glu^{21}]glucagon was synthesized on a chloromethylated polystyrene Merrifield resin (1% cross-linked with divinylbenzene—Lab Systems, San Mateo, CA). N^{α}-Boc-Thr(Bzl) was coupled to the resin as its cesium salt.[12] N^{α}-Boc-Asn and -Gln were coupled as their nitrophenyl esters in the presence of equimolar 1-hydroxybenzotriazole. The remainder of the N^{α}-Boc protected amino acids were coupled as their preformed symmetrical anhydrides.[13] The following side-chain protection was used: Asp(Chx), Ser(Bzl), Thr(Bzl), His(Tos), Trp(For), Lys(2,6-diClZ), and Tyr(2-BrZ). N^{α}-Boc protecting groups were removed by 50% trifluoroacetic acid in methylene chloride and neutralization was by 10% diisopropylethylamine in methylene chloride.

Deprotection and resin cleavage were performed in anhydrous HF at 0 °C for 60 min with added anisole and ethanedithiol to remove the Trp formyl group.[14] Purification was accomplished by dialysis against 2% aqueous acetic acid using Spectra-Por 6 dialysis-tubing with a molecular weight cut off of 1000 (Pierce Chemical Co., Rockford, IL), ion-exchange chromatography on a SP-Sephadex C-25 column with a linear gradient of 0–1 N NaCl in 6 M urea in 10% acetic acid, Sephadex G-10 column chromatography using 5% aqueous acetic acid as eluent solvent, and preparative reverse-phase HPLC on a Vydac 218TPB-16 Protein C18 column (25 × 250 cm) prepared by Perkin-Elmer using a 33–35% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) over 20 min at 20 mL/min. The final product was obtained (>97% purity) as determined by TLC (r_f 0.54 (*n*-BuOH/HOAc/H_2O/pyridine (60:12:48:60)); r_f = 0.58 (*i*-PrOH/NH_4OH/H_2O (3:1:1))), by reverse-phase HPLC on a 4.6 × 250 mm Vydac 218TPB-16 Protein C18 column (30% CH_3CN in 1% TFA 2 mL/min, k' = 5.41 (glucagon k' = 6.49)), by UV spectroscopy ϵ_{279} (30% HOAc) = 8719, and by amino acid analysis (Trp 0.90 (1), Lys 2.98 (3), His 1.04 (1), Asx 3.02 (3), Thr 2.88 (3), Ser 3.86 (4), Glx 4.17 (4), Gly 1.06 (1), Ala 1.02 (1), Val 0.91 (1), Met 0.96 (1), Leu 1.97 (2), Tyr 2.00 (2), Phe 2.09 (2). The final product could be crystallized in a form suitable for X-ray crystallography by the protocol for the crystallization of glucagon.[4]

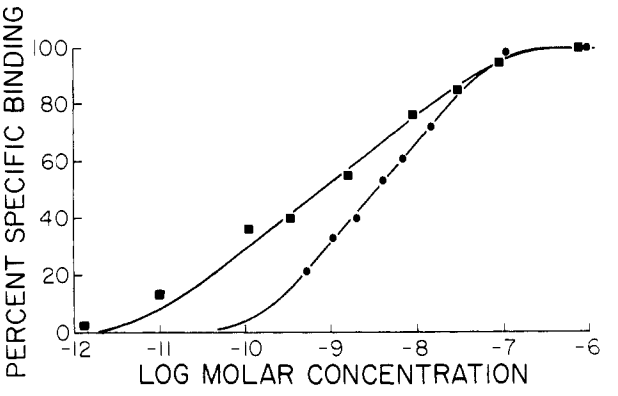

Figure 1. Competitive inhibition of ^{125}I–glucagon binding to liver plasma membrane receptor by glucagon (●) and [Lys^{17,18},Glu^{21}]glucagon (■). Each point represents the mean of three duplicate determinations.

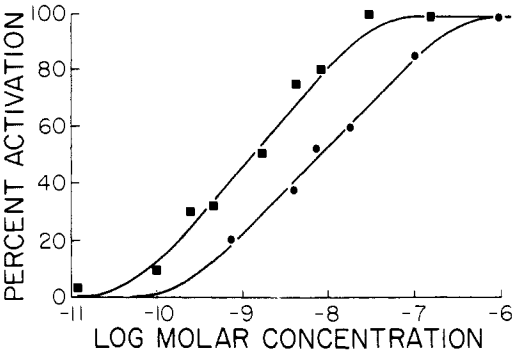

Figure 2. The log dose–response curve for glucagon (●) and [Lys^{17,18},Glu^{21}]glucagon (■) stimulation of adenylate cyclase activity in purified liver plasma membranes. Each point represents the mean of three determinations done in triplicate.

Chou–Fasman calculations indicate nearly an equal probability for β-structure $\langle P_a \rangle$ = 1.180 and $\langle P_b \rangle$ = 1.150 in the 19–27 region of glucagon. We, therefore, sought replacements which would increase α-helical probability and at the same time significantly decrease β-structure probability. The single replacement of Glu^{21} for Asp^{21} leads to an increased α-helical probability for the 19–27 region, $\langle P_a \rangle$ = 1.230 (from 1.180), with decreased β-structure probability, $\langle P_b \rangle$ = 1.070 (from 1.150). In addition, replacement of the adjacent Arg^{17} and Arg^{18} residues by Lys enhances α-helical probability of the 16–19 region of glucagon ($\langle P_a \rangle$ = 1.127 from 1.037) but decreases the β-structure probability ($\langle P_b \rangle$ = 0.7650 from 0.860).

The synthetic [Lys^{17,18},Glu^{21}]glucagon was 500% more potent than glucagon in the standard glucagon receptor binding assay[15] (Figure 1: IC_{50} = 0.70 nM (0.48–1.03 nM, 95% confidence limits) for [Lys^{17,18},Glu^{21}]glucagon; glucagon EC_{50} = 3.6 nM (2.0–4.4 nM, 95% confidence limits) in paired dose-response experiments). Furthermore, the analogue was a full agonist in the standard adenylate cyclase assay[16] in rat liver plasma membranes and was 700% more potent than the native hormone (Figure 2) (EC_{50} = 1.2 nM (0.9–1.6 nM, 95% confidence limits) for [Lys^{17,18},Glu^{21}]glucagon (EC_{50} = 8.4 nM (6.2–11.2 nM, 95% confidence limits) for glucagon). In the in vivo glucose release assay, the analogue was 100% the potency of glucagon. CD studies comparing [Lys^{17,18},Glu^{21}]glucagon to glucagon at the monomeric concentration 1 × 10^{-4} M in 0.2 M potassium posphate buffer (pH 9.2) indicates a significant increase in overall α-helical content ($[\theta]_{210}$ = −6224 (deg cm^2)/dmol compared to −4195 (deg cm^2)/dmol for glucagon).

These findings are significant in that this is the first series of modifications of glucagon in a region of the structure believed to be important primarily for receptor recognition that markedly

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increases glucagon potency in both the binding and adenylate cyclase assays.

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Transient Raman Evidence of the "One-Way" Cis to Trans Isomerization in the Lowest Excited Triplet State of 2-Styrylanthracene

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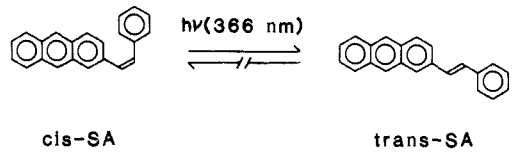
Elucidation of the mechanism of the cis-trans photoisomerization of olefins and related compounds has long been one of the central problems of photochemistry. The generally accepted scheme of photoisomerization assumes the so-called perpendicular excited singlet or triplet species as the key intermediate. Isomerization takes place after this species is diabatically converted to the perpendicular ground state which finally gives a mixture of the cis and trans isomers.[1] Recently, we found that this scheme is not applicable to the isomerization through the lowest excited triplet (T_1) states of some aromatic and conjugated polyene compounds. The direct and triplet-sensitized irradiation of 1-(2-anthryl)-3,3-dimethyl-1-butene in benzene resulted in a 100% "one-way" cis to trans isomerization.[2] The quantum yield for the cis to trans conversion far exceeded unity, implying a quantum chain process involving a long-lived intermediate that is most likely the trans T_1 species. From the observed nanosecond transient Raman spectra, it was proved that the photoexcitation of the 7-cis, 9-cis, and 11-cis isomers of retinal in hexane gave the same T_1 isomer as that produced from the all-trans form.[3] Similar results were obtained from the $T_n \leftarrow T_1$ absorption spectra of a group of anthrylethylenes.[4] In order to account for these photochemical and spectroscopic findings, we proposed a novel adiabatic isomerization mechanism in which the initially resulting cis T_1 species undergoes a rapid relaxation to the trans which is located at an energy minimum on the T_1 potential surface.

It seems important, at this stage, to focus our attention to the study of a simple prototypical molecule so that we are able to establish the concept of "one way" photoisomerization. In this paper, we wish to present new direct evidence that is based on transient Raman spectroscopy of 2-styrylanthracene (Scheme I; hereafter abbreviated as SA) and that is supported also by the photochemical data. Our transient Raman evidence is 2-fold. First, the same T_1 conformer is produced either from *trans*- or *cis*-SA by the photoexcitation, and, second, the Raman spectrum of this species is consistent with a trans structure.

The samples of *cis*- and *trans*-SA were prepared and purified as reported previously.[4] Transient resonance Raman spectra were obtained by using the third harmonic (355 nm, 10-Hz repetition, 5-ns pulsewidth) of a Q-switched Nd:YAG laser (Quanta-Ray DCR-2A) for pumping and the R-610 dye output (612 nm, 10 Hz, 5 ns) from a pulsed dye laser (Quanta-Ray PDL-1) for Raman probing. The probing pulse was delayed about 20 ns from the pumping pulse to avoid the interference from fluorescence. These two laser beams were spatially superimposed on a thin-film jet of the sample solution ($(1-2) \times 10^{-3}$ M in spectroscopic-grade benzene). The Raman scattered light was analyzed with a polychromator (Spex 1877) and detected with a gated (5 ns) intensified photodiode array detector (PAR 1420 and 1218). The spectral data were transferred to a minicomputer (DEC MINC-11) and processed. HPLC analysis of the sample solution showed that less than 10% of the *cis*-SA molecules photoisomerized during the Raman measurements. Raman spectra of crystalline SA in the ground state (S_0) were measured by using a He-Ne laser as the exciting source.

Scheme I



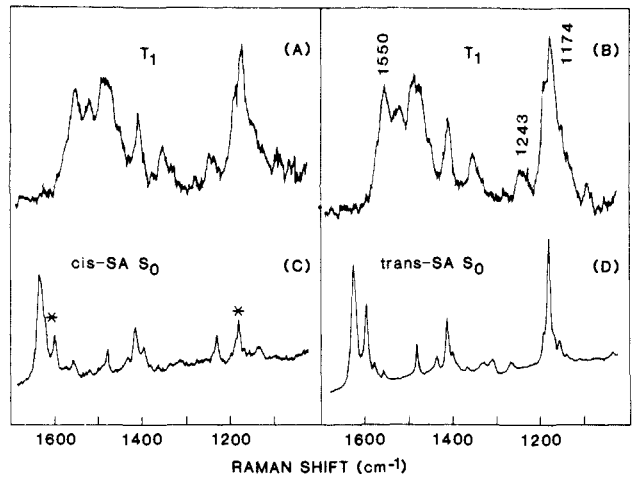


Figure 1. Transient T_1 Raman spectra obtained from benzene solutions of *cis*-SA (A) and *trans*-SA (B) and S_0 Raman spectra of crystalline *cis*-SA (C) and *trans*-SA (D). Asterisks in spectrum (C) mark the bands that might have small contributions from the trans molecules which were produced by the laser irradiation during the measurement.

It is already reported that *cis*-SA undergoes a 100% "one-way" isomerization to trans upon the direct photoexcitation at 366 nm (Scheme I).[4] In order to confirm that the isomerization takes place in the T_1 manifold and not in the S_1, both the direct and biacetyl (0.1 M) sensitized irradiation of *cis*-SA in deaerated benzene were performed at room temperature for various concentrations ranging from 1.31×10^{-4} to 1.57×10^{-3} M. In both cases, the observed quantum yield $\Phi_{c\rightarrow t}$ increased linearly with the concentration [*cis*-SA]; $\Phi_{c\rightarrow t} = \Phi_{ISC}(1 + k_q\tau_T[cis\text{-SA}])$, where Φ_{ISC} is the intersystem crossing quantum yield either for SA (direct irradiation) or for biacetyl (sensitized irradiation), k_q, the rate constant for the energy transfer from trans T_1 to cis S_0 giving cis T_1 to accomplish the quantum chain process, and τ_T, the lifetime of trans T_1 (190 μs in benzene at room temperature[4]). The value of $\Phi_{c\rightarrow t}$ was 6.8 and 17.8 at 1.57×10^{-3} M of [*cis*-SA] on the direct and sensitized irradiation, respectively, showing that the quantum chain process took place. The constant $k_q\tau_T$ was determined as 9.8×10^3 M^{-1} for the direct and 1.1×10^4 M^{-1} for the sensitized irradiation. Agreement between these two $k_q\tau_T$ values shows that the isomerization indeed occurs in the T_1 manifold.

The T_1 resonance Raman spectra and S_0 Raman spectra of *cis*- and *trans*-SA are shown in Figure 1. The two S_0 spectra (Figure 1C,D) are markedly different from each other, especially in the 1100-1300-cm^{-1} region where conformation-sensitive bands are located. On the other hand, T_1 spectra obtained from *cis*-SA (Figure 1A) and from *trans*-SA (Figure 1B) are identical within

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