Reputed Rat Scotophobin Prepared by a Solid-Phase Procedure Shown Invalid by Comparison with a Product Derived from a Classical Synthesis on the Basis of Physical and Biological Properties

GAY and RAPHELSON[1,2] found that extracts from the brains of rats trained to avoid the dark could upon injection into naive, dark-preferring rats or mice temporarily convert these recipient rodents to a dark-avoidance state. UNGAR[3], by use of a bioassay procedure, characterized the active principle as a peptide[4,5] and named it scotophobin[6]. Both the response of partially-purified rat scotophobin in mice and the peptide nature of the molecule was also confirmed by one of us[7]. UNGAR and his colleagues assigned various structures to natural rat scotophobin (two of which were synthesized[8,9]), but finally settled on H-Ser-Asp-Asn-Asn-Gln-Gln-Gly-Lys-Ser-Ala-Gln-Gln-Gly-Gly-Tyr-NH_2[10]. By a solid-phase procedure a product was obtained whose physical and biological properties were said to be identical with the natural material[11,12]. An extensive summary and a critical commentary on these efforts appeared simultaneously[13,14]. Although many questions remained with respect to the quality of the various analytical methods that led to the original structural formulation, as well as to the comparisons between natural and synthetic scotophobin[15], little attention was given to possible flaws in the solid-phase preparation itself[16,17]. Interestingly, this specific product had 'scotophobin activity' in the hands of several other research groups in that it temporarily reversed the dark-preference of naive animals for the following species: mice[18], goldfish[19–21], tenches[20], and roaches[22]. The negative findings and results of one group[23] were explained by UNGAR as a failure to follow his protocol[24], which is now available in greater detail[25]. When we showed the putative solid-phase rat scotophobin did not give bioassayable activity in rats[22], it was suspected that the synthetic compound was not identical with the natural product. To verify this point, RNA-free mixed oligopeptides were reisolated following an earlier procedure[5,7] and, like the original crude extracts of GAY and RAPHELSON[2], this material had a demonstrated physiological response in rats[26].

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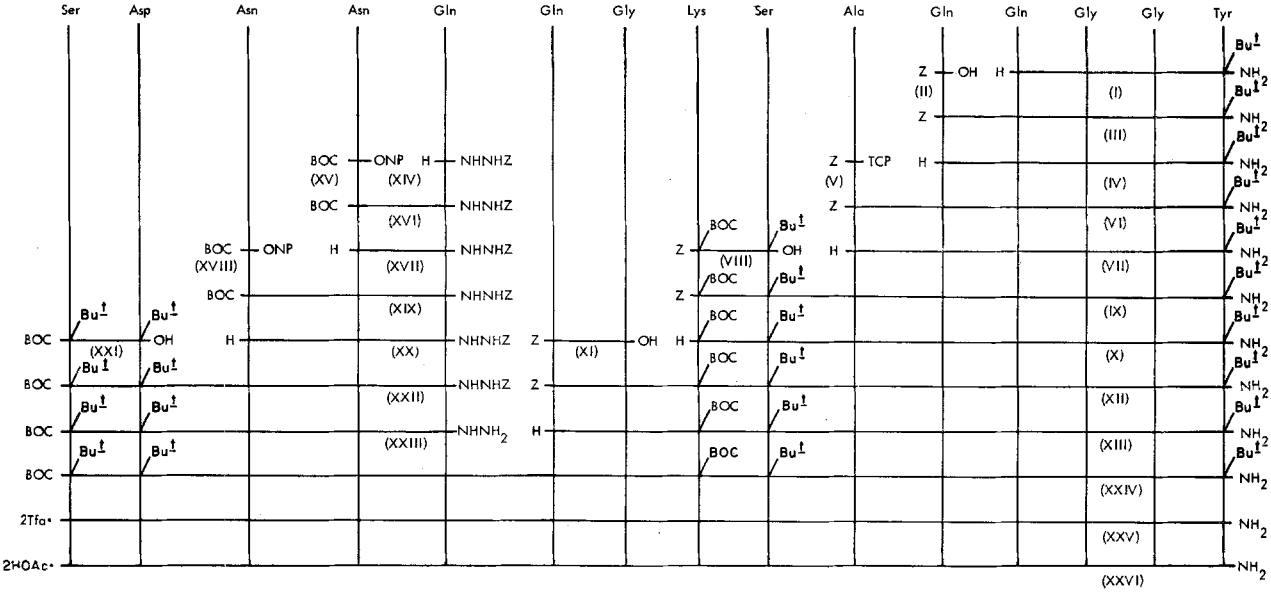

Fig. 1. Schematic diagram of the synthesis of a pentadecapeptide with the structure assigned to rat scotophobin: BOC, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bu^t, *t*-butyl ether; OBu^t, *t*-butyl ester; ONP, *p*-nitrophenyl ester; TCP, 2,4,5-trichlorophenyl ester; HOAc, acetate; and Tfa, trifluoroacetate.

We now report the synthesis by classical means and the resulting lack of biological activity for a pentadecapeptide having the structure considered by UNGAR[27] to be identical with rat scotophobin. In the new preparation, each intermediate, wherever possible, was isolated and purified, then characterized by various spectroscopic methods (infrared, nuclear magnetic resonance, and ultraviolet) and analytical techniques (amino acid analysis, thin layer chromatography in several solvent systems, and microcombustion). The route began by joining L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (I)[9] to N$^{\alpha}$-benzyloxycarbonyl-L-glutamine (II) by a mixed carbonic anhydride-ethyl morpholine combination so as to give N$^{\alpha}$-benzyloxycarbonyl-L-glutaminyl-L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (III), mp 225°. Removal of the benzyloxycarbonyl protecting group by catalytic hydrogenation afforded the pentapeptide amine (IV), which was coupled to 2, 4, 5-trichlorophenyl N$^{\alpha}$-benzyloxycarbonyl-L-alaninate (V) to yield the hexapeptide N$^{\alpha}$-benzyloxycarbonyl-L-alanyl-L-glutaminyl-L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (VI), mp 224°. Deblocking in the usual fashion formed the amine (VII), and coupling with N$^{\alpha}$-benzyloxycarbonyl-N$^{\varepsilon}$-*t*-butyloxycarbonyl-L-lysyl-*O*-*t*-butyl-L-serine (VIII) furnished N$^{\alpha}$-benzyloxycarbonyl-N$^{\varepsilon}$-*t*-butyloxycarbonyl-L-lysyl-*O*-*t*-butyl-L-seryl-L-alanyl-L-glutaminyl-L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (IX), mp 258°. Hydrogenolysis produced the octapeptide amine (X), mp 234°, which on combination with N$^{\alpha}$-benzyloxycarbonyl-L-glutaminylglycine (XI), mp 184°, via a mixed anhydride reagent gave N$^{\alpha}$-benzyloxycarbonyl-L-glutaminylglycyl-N$^{\varepsilon}$-*t*-butyloxycarbonyl-L-lysyl-*O*-*t*-butyl-L-seryl-L-alanyl-L-glutaminyl-L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (XII), mp 258°. Removal of the benzyloxycarbonyl function in the usual way resulted in the formation of the decapeptide amine (XIII).

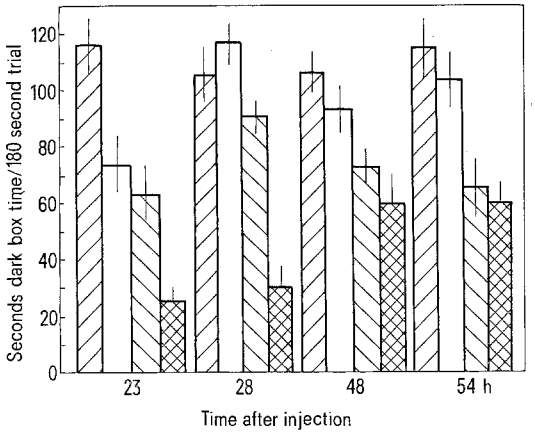

Fig. 2. Transfer bioassay on goldfish of classically synthesized pentadecapeptide with the structure previously reported to be identical with rat scotophobin. In all cases fish were injected i.p. with 50 µl of vehicle ▨, control; □, peptide 5.0 ng; ▧, 50.0 ng; ▩, 500.0 ng. All preparations were coded and their identity unknown to the person administering the injection or testing fish. Decoding and analysis was done after the assay was completed. Recipient screening and injection has been described[22]. At the indicated times after injection, dark box time of individual recipients out of a total of 180-sec test period was recorded and after an intertrial interval of 60 sec, a 2nd 180-sec test was run. For all dose levels, 4 groups of 5 fish were tested. Data are expressed as mean of the 4 means for each analysis. Highly significant activity for the 500.0 ng dose is exhibited at 23 and 28 h after injection.

The remaining portion of the sequence was prepared by a stepwise procedure. L-Glutaminyl N^{2}-benzyloxycarbonylhydrazide (XIV), mp 160°, was reacted with *p*-nitrophenyl N$^{\alpha}$-*t*-butyloxycarbonyl-L-asparaginate (XV) to afford N$^{\alpha}$-*t*-butyloxycarbonyl-L-asparaginyl-L-glutaminyl N^{2}-benzyloxycarbonylhydrazide (XVI), mp 197°. Stirring with trifluoroacetic acid cleaved the *t*-butyloxycarbonyl moiety, and the resulting salt was converted into L-asparaginyl-L-glutaminyl N^{2}-benzyloxycarbonylhydrazide (XVII). Retreatment with *p*-nitrophenyl N$^{\alpha}$-*t*-butyloxycarbonyl-L-asparaginate yielded N$^{\alpha}$-*t*-butyloxycarbonyl-L-asparaginyl-L-asparaginyl-L-glutaminyl N^{2}-benzyloxycarbonylhydrazide (XIX), mp 202°. Addition of trifluoroacetic acid formed the tripeptide amine salt, which was neutralized to furnish the amine (XX). A coupling with N$^{\alpha}$-*t*-butyloxycarbonyl-*O*-*t*-butyl-L-seryl-*β*-*t*-butyl-L-aspartic acid (XXI) by a mixed anhydride reagent produced N$^{\alpha}$-*t*-butyloxycarbonyl-*O*-*t*-butyl-L-seryl-*β*-*t*-butyl-L-aspartyl-L-asparaginyl-L-asparaginyl-L-glutaminyl N^{2}-benzyloxycarbonylhydrazide (XXII), mp 221°. Deletion of the benzyloxycarbonyl group generated the free hydrazide (XXIII), which was changed into the corresponding azide by treatment with *t*-butyl nitrite. Addition of the decapeptide amine (XIII) afforded N$^{\alpha}$-*t*-butyloxycarbonyl-*O*-*t*-butyl-L-seryl-*β*-*t*-butyl-L-aspartyl-L-asparaginyl-L-asparaginyl-L-glutaminyl-L-glutaminyl-

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Thin layer chromatography of the pentadecapeptide[a] synthesized by classical methods and one previously prepared by solid-phase procedures and reputed to have the same structure

Plate conditions[b]	Solvent system (volume:volume)	Classical	Solid-phase	
		Chicago-Seattle	Chicago[39]	Houston[11–13]
1. Activated:	*n*-BuOH:EtOH:HOAc:H_2O (8:2:1:3)	0.25–0.30	—	0.58
Unactivated:	*n*-BuOH:EtOH:HOAc:H_2O (8:2:1:3)	0.00	—	—
2. Activated:	Pyr:MEK:HOAc:H_2O (15:70:2:15)	0.00	—	—
Unactivated:	Pyr:MEK:HOAc:H_2O (15:70:2:15)	0.00	—	—
3. Activated:	EtOH:Pyr:HOAc:EtAc:H_2O (5:1:2:2:2)	0.25	—	—
Unactivated:	EtOH:Pyr:HOAc:EtAc:H_2O (5:1:2:2:2)	0.28	—	—
4. Unactivated:	H_2O:90% HCO_2H (100:1.5)	0.06	0.07	0.16
5. Unactivated:	C_6H_6:HOAc (9:1)	0.31	0.12	0.18

[a] H-Ser-Asp-Asn-Asn-Gln-Gln-Gly-Lys-Ser-Ala-Gln-Gln-Gly-Gly-Tyr-NH_2. [b] Silica gel plates were used for 1, 2, and 3. Polyamide plates were used for 4 and 5 where the compounds analyzed were the dansyl derivatives.

glycyl-N$^{\varepsilon}$-*t*-butyloxycarbonyl-L-lysyl-*O*-*t*-butyl-L-seryl-L-alanyl-L-glutaminyl-L-glutaminylglycylglycyl-*O*-*t*-butyl-L-tyrosinamide (XXIV), mp 250°. Stirring with trifluoroacetic acid-anisole removed all of the remaining *t*-butyloxycarbonyl and *t*-butyl ester protecting groups and yielded the pentadecapeptide salt (XXV). The compound was passed through an ion-exchange resin column (AG-1-X2) to form the equivalent acetate salt, which on lyphilization produced a fluffy white solid (XXVI). This material possessed a correct amino acid and ammonia content as determined by hydrolysis and was optically pure via a leucine aminopeptidase digestion. Thus, it may be assumed the peptide obtained here is indeed L-seryl-L-aspartyl-L-asparaginyl-L-asparaginyl-L-glutaminyl-L-glutaminylglycyl-L-lysyl-L-seryl-L-alanyl-L-glutaminyl-L-glutaminylglycylglycyl-L-tyrosinamide. We restress that the various intermediates were characterized in one or more ways as each step proceeded during the overall synthetic operation. Based on the final analytical methods, the peptide is believed to be at least 90% pure. The complete synthetic scheme is summarized in Figure 1.

Permethylation of the free peptide gave a derivative that was subjected to a mass spectral analysis by Dr. NICHOLAS C. LING, Salk Institute. The fragmentation patterns confirmed the presence of the last 8 residues of the described sequence. However, structural information beyond this point was uncertain, for extensive degradation of the remaining lower molecular weight ions interfered with a clear-cut interpretation of the spectrum. Treatment with trypsin did not cleave the compound into 2 smaller units, as claimed for both natural and solid-phase scotophobin. It must be recalled that the enzyme/substrate ratio (4:1) used by the Houston workers was 200 times greater than normally employed in such studies. Along other lines, our product is stable in solution over prolonged periods; by contrast, the solid-phase putative scotophobin preparation is labile in water. Natural scotophobin is, of course, isolated by repeated chromatography in a variety of aqueous systems, so the reported decomposition of the solid-phase peptide is rather puzzling. The possibility that the solid-phase product undergoes a facile deamidation[28,29] or contains rearranged β-aspartyl links[30,31], pyroglutamyl residues[32], and cyano amino acids[33,34], must be taken into consideration and could well account for the observed instability. If true, then such a substance cannot be identical with natural scotophobin.

The classical synthetic work described here was tedious and time consuming, but clearly indicates the final compound possesses the structure attributed to it and is homogeneous by generally accepted concepts. By contrast, solid-phase synthesis beyond the decapeptide level is now realized to generate a broad spectrum of materials composed of truncated, deleted, and mixed peptides, in which the desired sequence may constitute only a fraction of the last operational step[35,36]. Naturally, the restriction of the analysis to a single amino acid assay or a tryptic digestion, as reported by the Houston group, affords little information as to the presence or absence of rearranged sequences, the extent of racemization, etc. Such data might be provided by a mass spectral study on the solid-phase peptide. However, no work along these lines has been reported in the past. The biological comparison of the solid-phase material with the natural peptide was done on a weight basis, which is particularly misleading, because apart from error peptides (that may not be inert), account was not taken of inorganic salt and solvent content[37,38].

We assayed our product in rats, mice, goldfish, and roaches, while Professor G. UNGAR tested the same compound in mice. In both laboratories this peptide was ineffective in mice; however, we agreed with Professor UNGAR that the material synthesized by the solid-phase method[11,12] and reputed to be identical with rat scotophobin caused a response in mice[13,18,22,25]. Further, the classically-synthesized peptide was inactive with roaches although the peptide synthesized by the solid-phase method[11,13] was active for roaches[22]. It was shown previously that the solid-phase peptide was active for goldfish[19,39], and as can be seen from Figure 2, our compound is also active in goldfish. Yet, the present product is not identical with natural goldfish scotophobin because the Rf's of the dansylated peptides are different. This bit of work is based on the isolation of pure goldfish scotophobin[40], whose sequence is under current study. Lastly, neither the classical nor the solid-phase materials are the same as natural rat scotophobin because the solidphase product is inert for rats[22], and we can now add that the classical peptide is also unable to produce a response in rats. This double failure is definitive in dismissing either compound as having the same composition as rat scotophobin since: a) the original peptide was isolated from rat brains, b) the crude brain extracts from dark-avoiding rats can transfer dark-avoidance to rat[2], and c) RNA-free mixed oligopeptides from dark-avoidance trained rats, but not from yoked controls, transfer dark-avoidance to rats[26] and mice[7,26].

The true structural identity of the solid-phase peptide should be determined because it does possess biological activity for a number of species[13,18-21] including one invertebrate[22], and therefore is a compound of considerable interest for neurobiochemical studies[41]. Our classical peptide must be relegated to the scotophobin-like category, although it possesses the sequence previously attributed to rat scotophobin. It is therefore quite important to reisolate natural rat scotophobin and to carefully redefine its primary structure. After these steps have been taken and the work confirmed by independent laboratories, then one can with greater confidence approach the problem of synthesizing a product identical with rat scotophobin.

In conclusion, we summarize in the Table the differences between the chromatographic values for the peptide prepared by the solid-phase procedure[11-13] and reputed to be identical to rat scotophobin with a

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compound synthesized by an alternative classical method. The material obtained by the Texas workers cannot be identical with natural rat scotophobin because it lacks biological activity in rats, while natural rat scotophobin affords such a response. As a result, the latest structure attributed to scotophobin is in error[42].

Zusammenfassung. Eine Synthese von Scotophobin mit klassischen Methoden ergab ein Produkt, das sich vom natürlichen Peptid hinsichtlich seiner biologischen, chromatographischen und physiologischen Eigenschaften unterschied. Es wird daraus gefolgert, dass die für das natürliche Produkt vorgeschlagene Strukturformel nicht korrekt ist.

HELENE N. GUTTMAN and BORIS WEINSTEIN, RITA M. BARTSCHOT and PETER S. TAM

Department of Biological Sciences, University of Illinois at Chicago Circle, Chicago (Illinois 60680, USA), and Department of Chemistry, University of Washington, Seattle (Washington 98195, USA), 6 November 1974.

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Reversal of Dominance in the Competition Between *Drosophila nasuta* and *Drosophila neonasuta*

Interspecific competition studies involving chromosomally polymorphic and monomorphic populations of *Drosophila nasuta* and *Drosophila neonasuta* have revealed that irrespective of the strains in competition, *D. nasuta* supplants *D. neonasuta*. Here in all instances the initial frequency of the two competing species was 1:1 (25:25)[1].

In an extention of these tests interspecific competition studies were made with the initial frequency as 1 *D. nasuta* : 4 *D. neonasuta* (10:40). Simultaneously the interspecific competition process with 1:1 ratio was also followed in these 2 species. Serial transfer technique of AYALA[2] was adopted to maintain the experimental populations; 4 replicates were made for each set of experiments. The entire experiment was conducted at 21°C. The females of these 2 species are morphologically indistinguishable. However, the males can be differentiated from one another. Males of *D. nasuta* have complete silvery frons while the males of *D. neonasuta* have silvery markings around the frontal orbits only. Therefore, at each census the number of males of each species and the total population size were recorded. The cultures were maintained until the elimination of any one of the competing species.

Figures 1 and 2 illustrate the dynamics of the interspecific competition of *D. nasuta* and *D. neonasuta* initiated with 1:1 and 1:4 frequencies respectively. *D. nasuta* eliminates *D. neonasuta* when the initial ratio was 1:1 but that itself faces extinction when the founder population was in the ratio of 1:4. The species performances as measured by their mean number of males and the average population size maintained during competition are presented in the Table. Persual of this Table indicates the reversal of dominance of the 2 contesting species in the 2 sets of experiments.

Here the outcome of the competition is determined by the initial frequencies of the competing species. The initial advantage gained by the higher frequency of the founder population has made *D. neonasuta* to outvie *D. nasuta*.

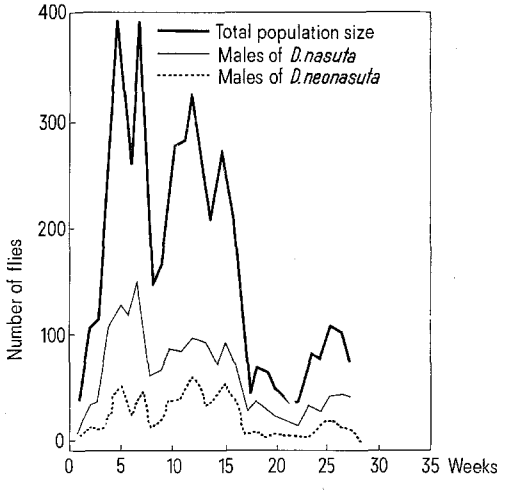

Fig. 1. Total population size and the number of males of *D. nasuta* and *D. neonasuta* during competition started with 1:1 frequency.

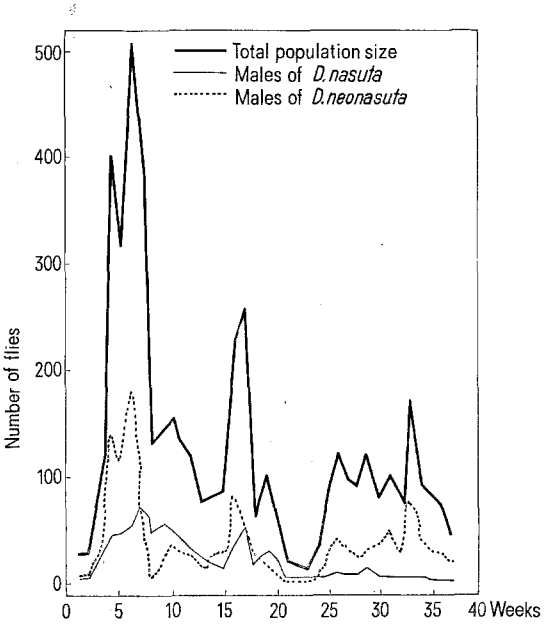

Fig. 2. Total population size and the number of males of *D. nasuta* and *D. neonasuta* during competition started with 1:4 frequency.

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