Synthesis and melanotropic activity of γ-melanotropin

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Abstract. γ-MSH, a third melanotropic sequence in the *N*-terminal cryptic portion of the ACTH/β-LPH precursor molecule pro-opiocortin, was synthesized by the fragment condensation approach. The dodecapeptide H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH was tested for melanocyte-stimulating activity on the skin of the lizard *Anolis Carolinensis* and was found to possess only 2.5×10^{-4} of the activity of α-MSH.

Introduction

During the process of biosynthesis both corticotropin (ACTH) and β-lipotropin (β-LPH) form part of a common precursor molecule named pro-opiocortin[1,2]. Recently *Nakanishi* et al.[3] elucidated the nucleotide sequence of the complementary DNA, corresponding with the messenger RNA chain coding for this ACTH/β-LPH precursor protein and predicted the amino acid sequence of the complete pro-opiocortin molecule. Their results indicate that the precursor protein consists of repetitive units and includes a third melanotropic sequence in its *N*-terminal cryptic portion; *Nakanishi* et al. suggested the name "γ-melanotropin", γ-MSH. The structure proposed for the fragment which on both sides is flanked by a pair of basic amino acid residues, is H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH (*cf.* α-MSH: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2).

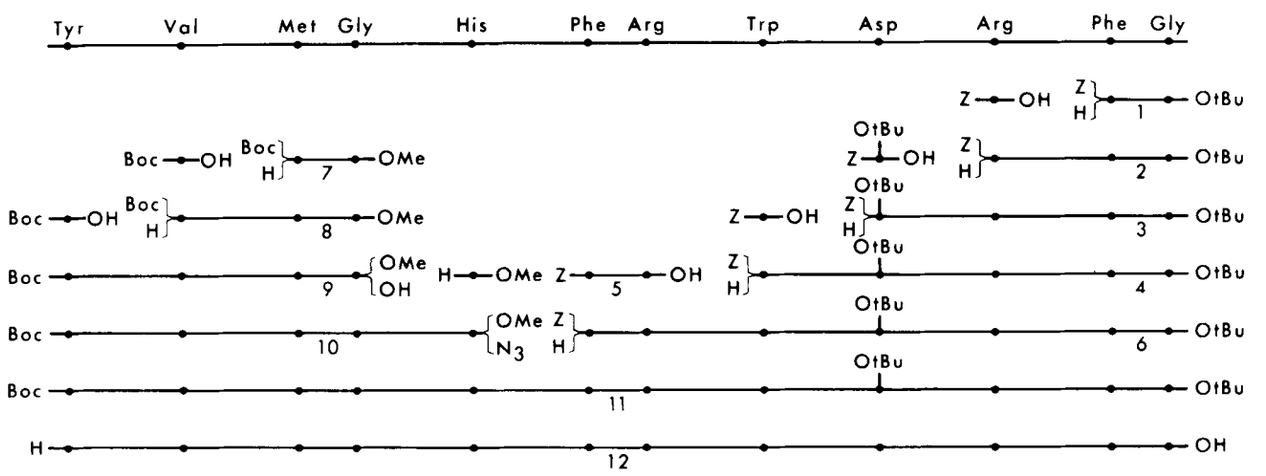
We were interested in this γ-MSH in view of the hypothesis of *De Wied*[4] that the pituitary and/or brain may contain neuropeptides, which are involved in motivational, learning and memory processes, and are more potent and specific than the known ACTH and β-LPH fragments. This hypothesis has found support in a recent publication[5]. We decided to synthesize the sequence proposed for γ-MSH, but without *N*- or *C*-terminal modification as found in α-MSH, *i.e.* no acetyl group on the N^{α} of tyrosine nor a phenylalanine amide at the carboxyl terminus.

In this paper we describe the synthesis of the dodecapeptide γ-MSH by the fragment condensation approach and its melanocyte-stimulating activity relative to the potent melanotropic peptide α-MSH. Results of behavioural tests and a comparison of γ-MSH with β_h-endorphin [*i.e.* β_h-LPH-(61–91)] and ACTH (1–24) will be published elsewhere[6].

In the meantime the solid phase synthesis of γ-MSH and three related peptides has been published together with some of their biological activities[7].

Strategy and description of the synthesis

We used the fragment condensation approach for the synthesis of the desired dodecapeptide for reasons described earlier[8]. The protecting groups for the *N*- and *C*-terminus and the β-carboxyl group of the aspartic acid residue were *tert*-butyl derived in order to be able to use a mild acid treatment in the final stage of the synthesis. The guanidino function of Arg was protected by protonation, the imidazole moiety of His and the side-chain of Tyr were not protected. Reaction of His via Z-His-N_3 with the C-terminal heptapeptide gave a product that was difficult to purify. Therefore, the approach as outlined in Fig. 1 was chosen. In this



removal of one of these protecting functions the letter a is added, e.g. *Z-Tyr-Val-Met-Gly-OMe will be referred to as* **9** *and the corresponding tetrapeptide acid as* **9a**.

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approach, a pentapeptide, **10** and a heptapeptide, **6**, were synthesized and coupled via an azide activation of His which is the *C*-terminal amino acid residue of **10** (Fig. 1). The *C*-terminal heptapeptide **6** was built up from a di- and a pentapeptide. The pentapeptide **4** was synthesized starting from the known dipeptide Z-Phe-Gly-OtBu[9] by a stepwise procedure using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as the coupling reagents[10]. The α-amino group was liberated at each stage by hydrogenolytic removal of the benzyloxycarbonyl (Z) function. Z-Phe-Arg-OH, **5**[11] [obtained by the coupling of Z-Phe-OH and H-Arg-OH via a mixed anhydride reaction] was coupled with H-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HCl in DMF using DCC and HOBt. The *N*-terminal tetrapeptide ester **9** was built up stepwise using DCC and HOBt. Saponification of **9** in dioxane/water (9 : 1, v/v) with 2.2 equivalents of NaOH gave the corresponding tetrapeptide acid **9a**. Again, DCC and HOBt were used for the synthesis of the pentapeptide ester **10**; conversion of this ester into the hydrazide provided the desired *N*-terminal fragment. The C-terminal heptapeptide **6** was hydrogenated and the resulting product was purified by chromatography on silica (Merck Fertigsäule) prior to acylating with Boc-Tyr-Val-Met-Gly-His-N_3. The crude product **11** was treated without further purification with TFA/H_2O (9 : 1, v/v) in the presence of anisole and di-*tert*-butyl sulfide under a N_2 blanket. After conversion of the trifluoroacetate salt into the acetate salt, the free dodecapeptide **12** was purified by chromatography on silica (Merck Fertigsäule).

The synthesis of the reference compound, the tridecapeptide α-MSH, was carried out approx. 6 years ago according to the method described by *Schwyzer* et al.[12]. Ac-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-NH_2 was treated with TFA and the crude product purified by counter current distribution (Craig partition). Re-purification just before testing provided α-MSH of high quality.

The final products were checked for purity by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), amino acid analysis and L-amino acid oxidase (LAO) digestion[8].

Assay of melanotropic activity

The test of *Burgers*[13] was used for the determination of the melanocyte-stimulating activity of γ-MSH. In this *in vitro* method, stimulated darkening of the skin of the lizard *Anolis Carolinensis* is evaluated visually. Synthetic α-MSH was used as the standard, and its activity was set at 1.00. The experiments were carried out in duplicate.

The activity of γ-MSH was found to be 2.5×10^{-4} of that of α-MSH (both on a weight and molar basis when the latter is also corrected for peptide content).

Experimental section

The purity of the amino acid derivatives and peptides was checked by thin-layer chromatography (TLC) on Merck silicagel plates (F.254, 0.25 mm) using the following solvent systems (ratios are v/v):

a) methylene chloride/methanol/water = 70 : 30 : 5
b) toluene/ethanol = 4 : 1
c) 1-butanol/pyridine/acetic acid/water = 16 : 3 : 1 : 4
d) 1-butanol/pyridine/acetic acid/water = 8 : 3 : 1 : 4
e) 1-butanol/acetic acid/water = 4 : 1 : 1

Methods for detection of components on TLC plates and the description of other standard methods are the same as reported previously[8].

A. Synthesis of Z-Phe-Arg-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu (6)

Z-Arg-Phe-Gly-OtBu·HCl **(2)**

12.2 g (39.5 mmol) of Z-Arg-OH were dissolved in 70 ml of dimethylformamide (DMF) and 14.1 ml of 2.8 N HCl/DMF. To this solution were added successively at −20°C 5.4 g (1 equiv.) of HOBt, 12.4 g (39.5 mmol) of H-Phe-Gly-OtBu·HCl [obtained after hydrogenation of the corresponding Z-Phe-Gly-OtBu[9] in the presence of 1 equiv. of HCl] and 5.0 ml of *N*-ethylmorpholine (NEM) in 50 ml of DMF and 9.1 g (1.1 equiv.) of DCC in 20 ml of DMF. After stirring for 15 min at −20°C and 18 h at room temperature, the reaction mixture was filtered and the filtrate evaporated to dryness. The oily residue was dissolved in 2-BuOH/$CHCl_3$ (2 : 3, v/v) and the solution extracted with 1N HCl solution and H_2O. After drying (Na_2SO_4) the solution was evaporated to dryness to give an oil in a nearly quantitative yield. $[\alpha]_D^{20}$ −16.9° (*c*1, DMF). TLC: R_f 0.69 (a).

*Z-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX** **(3)**

12.9 g (40.0 mmol) of Z-Asp(OtBu)-OH and 5.4 g (1 equiv.) of HOBt were dissolved in 50 ml of DMF. To this solution were added at −20°C 17.2 g (33.8 mmol) of H-Arg-Phe-Gly-OtBu·2HCl [obtained after hydrogenation of 24.1 g (39.8 mmol) of Z-Arg-Phe-Gly-OtBu·HCl in DMF with 1 equiv. of HCl] and 4.26 ml of NEM in 80 ml of DMF followed by 8.3 g (1 equiv.) of DCC in 20 ml of DMF. After stirring for 15 min at −20°C and 18 h at room temperature, the reaction mixture was filtered and the filtrate was evaporated to dryness. The oily residue was dissolved in ethyl acetate (EtOAc) and the organic phase was extracted with H_2O, 1 N HCl solution, 5% $NaHCO_3$ solution and again with H_2O.

After drying (Na_2SO_4), the solution was evaporated to dryness. Precipitation from EtOAC/pet. ether gave 26.6 g (nearly quantitative yield) of the tetrapeptide **3**. $[\alpha]_D^{20}$ −18.7° (*c*1, DMF). TLC: R_f 0.79(e).

Z-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX **(4)**

3.1 g (9.1 mmol) of Z-Trp-OH and H-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX (obtained after hydrogenation of 7.76 g (10.0 mmol) of the corresponding Z-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX in DMF with 1 equiv. of HCl) were coupled using DCC (1.1 equiv.) and HOBt (1.5 equiv.). DCU was removed by filtration and the filtrate was evaporated to dryness. The oily residue was dissolved in EtOAc and extracted with 5% $KHSO_4$ solution, 5% $NaHCO_3$ solution and saturated NaCl solution. After drying (Na_2SO_4) the solution was evaporated to dryness. Precipitation from EtOAc/pet. ether gave 7.3 g (approx. 76.0%) of the pentapeptide derivative.
$[\alpha]_D^{20}$ −34.0° (*c*1, DMF). TLC: R_f 0.78(a).

Z-Phe-Arg-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX **(6)**

2.9 g (3.0 mmol) of compound **4** were hydrogenated in DMF with 1 equiv. of HCl and Pd/C (10%) as the catalyst. After removal of the catalyst by filtration, 1.5 g (3.0 mmol) of Z-Phe-Arg-OH[11] were added to the filtrate. The coupling was analogous to the one described for **4**. After removal of the DCU by filtration, the filtrate was evaporated to dryness. The residue was precipitated from EtOAc/pet. ether and then the crude product was purified by

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* It was found by analytical, capillary isotachophoresis[14] that in addition to HCl the peptide also contained a small amount of HOBt[15]. This is also the case for peptides **4**, **6** and **6a**. After chromatography on silica using the solvent system 1-BuOH/pyridine/HOAc/H_2O (in **6** and **6a**) a small amount of acetic acid (HOAc) was also found. In reactions carried out with peptides **3**, **4**, **6** and **6a** the number of mmoles was calculated from the molecular mass and including the theoretical amount of hydrogen chloride (not corrected for HOBt or HOAc present). Yields in % are approximate figures.

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chromatography on silica (Merck Fertigsäule) with solvent system 1-BuOH/pyridine/HOAc/H_2O = 32:3:1:4, by volume. Yield 2.3 g; $[\alpha]_D^{20}$ −27.7° (*c*1, DMF). Amino acid analysis: Asp 1.06, Gly 1.00, Phe 1.97, Arg 1.99, Trp 0.62. (Trp is partially destroyed under the hydrolysis conditions). Peptide content 88.6%. TLC: R_f 0.73 (a).

B. Synthesis of Boc-Tyr-Val-Met-Gly-His-N_2H_3 (10a)

Boc-Val-Met-Gly-OMe (**8**)

15.5 g (72.3 mmol) of Boc-Val-OH and 20.0 g (72.3 mmol) of H-Met-Gly-OMe·HCl [obtained after treatment of Boc-Met-Gly-OMe, 7[16] with HCl in CH_2Cl_2] and 9.13 ml of NEM in 200 ml of DMF were coupled using 1.0 equiv. of DCC and 1.0 equiv. of HOBt. After working up as described for compound **4**, crystallization from EtOAc/pet. ether gave 24.1 g (75.0%) of the tripeptide derivative **8**. M.p. 111–112°C, $[\alpha]_D^{20}$ −17.6° (*c*1, DMF). TLC: R_f 0.55 (b).

Boc-Tyr-Val-Met-Gly-OMe (**9**)

15.2 g (54.0 mmol) of Boc-Tyr-OH and 20.3 g (54.0 mmol) of H-Val-Met-Gly-OMe·HCl (obtained after treatment of the corresponding Boc compound with HCl in CH_2Cl_2) and 6.8 ml of NEM in 300 ml of DMF were coupled and worked up as described for compound **8**. The crude product was purified by chromatography on silica (Merck Fertigsäule) with solvent system toluene/ethanol = 9:1, v/v. Yield, after crystallization from EtOH/ether, 7.6 g (23.4%). M.p. 193°C (dec.), $[\alpha]_D^{20}$ −6.4° (*c*1, DMF). TLC: R_f 0.50 (b).

Boc-Tyr-Val-Met-Gly-OH (**9a**)

4.4 g (7.3 mmol) of Boc-Tyr-Val-Met-Gly-OMe in 100 ml of dioxane H_2O (9:1, v/v) were treated with 8.1 ml of 2.0 N NaOH for 15 min at room temperature. The pH was then adjusted to 7 with 4N HCl and the dioxane was evaporated. EtOAc was added and the pH of the mixture was adjusted to 2 with 4N HCl. The organic phase was separated and extracted with saturated NaCl solution. After drying (Na_2SO_4), the solution was evaporated to dryness. The residue was dissolved in MeOH and **9a** precipitated with EtOAc/ether. Yield 4.6 g (approx. 100%); $[\alpha]_D^{20}$ −5.5° (*c*1, DMF). TLC: R_f 0.45 (a).

Boc-Tyr-Val-Met-Gly-His-N_2H_3 (**10a**)

3.4 g (6.0 mmol) of Boc-Tyr-Val-Met-Gly-OH and 1.4 g (5.6 mmol) of H-His-OMe·2HCl in 40 ml of DMF with 1.5 ml of NEM were coupled using DCC (1.1 equiv.) and HOBt (2.0 equiv.). After removal of the DCU by filtration, the filtrate was evaporated to dryness. The oily residue was dissolved in EtOAc/H_2O (4:1, v/v) and the organic phase was extracted with 5% $NaHCO_3$ solution and saturated NaCl solution. After drying (Na_2SO_4) the solution was evaporated to dryness to give an oil in 75.0% (3.0 g) yield. 2.9 g (4.0 mmol) of this crude product were dissolved in 50 ml of MeOH and 4.0 ml of $N_2H_4 \cdot H_2O$ were added. The reaction mixture was stirred for 18 h at room temperature. Ether was then added and the precipitate was filtered. The residue was stirred with water, the precipitate filtered, washed with water and dried. Yield 1.3 g (45.0%), m.p. 219–220°C (dec.), $[\alpha]_D^{20}$ −2.6° (*c*1, DMF). Amino acid analysis: His 0.99, Gly 1.02, Val 0.98, Met 0.95, Tyr 1.06. Peptide content 93.5%. TLC: R_f 0.43 (c).

C. Synthesis of H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Pe-Gly-OH, γ-MSH (12)

H-Phe-Arg-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX (**6a**)

2.0 g (approx. 1.5 mmol) of Z-Phe-Arg-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX were hydrogenated in DMF containing 3 equiv. of HCl and with Pd/C (10%) as the catalyst. After removal of the catalyst, EtOAc was added to the filtrate and the precipitate was filtered, washed with EtOAc, and dried. Yield 1.6 g (approx. 86%). The product was purified by chromatography on silica (Merck Fertigsäule) with solvent system c. Yield of chromatographically pure H-Phe-Arg-Trp-Asp(OtBu)-Arg-Phe-Gly-OtBu·HX was 380 mg.
Amino acid analysis: Trp 0.91, Arg 1.91, Asp 1.00, Gly 1.00, Phe 2.08. Peptide content 76.9%. TLC: R_f 0.36 (c).

H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH, γ-MSH, (**12**)

209 mg (0.3 mmol) of Boc-Tyr-Val-Met-Gly-His-N_2H_3 in 10 ml of DMF were treated with 0.59 ml 1.5 N HCl in DMF at 0°C. The hydrazide was converted into the azide by the addition of 0.041 ml (0.3 mmol) of isopentyl nitrite and stirring the solution for 15 min at −15/−20°C. The reaction mixture was then neutralized with 0.13 ml of NEM, and a solution of **6a** (350 mg, approx. 0.3 mmol) in 10 ml of DMF was subsequently added. The pH of the solution was adjusted to 7.2 with NEM. After 102 h at 0°C the reaction mixture was concentrated to approx. 2 ml and poured into EtOAc (40 ml). The precipitate was filtered, washed with EtOAc and dried. Yield of not homogeneous peptide **11**: 497 mg.
Without purification, peptide **11** was treated with 5 ml of trifluoroacetic acid/water (9:1, v/v), under N_2, and in the presence of anisole and di-*tert*-butyl sulfide. After conversion of the trifluoroacetate salt into the acetate salt, the crude product was purified by chromatography on silica (Merck Fertigsäule) with the solvent system 1-BuOH/pyridine/HOAc/H_2O = 20:3:1:4, by volume. Yield 74 mg, homogeneous upon TLC, R_f 0.30 (d). Amino acid analysis: Asp 1.02, Gly 2.07, Val 0.93, Met 1.02, Tyr 0.97, Phe 2.00, His 0.99, Trp 0.83. Peptide content 80.8%. LAO digestion: no racemization of any importance was found. Acetic acid content determined isotachophoretically[15]: 8.2%; no other anions were detected. $[\alpha]_D^{20}$ −38.0° (*c*0.5, 10% HOAc); Lit.[7]: $[\alpha]_D^{23}$ −33.4° (*c*1, 1% HOAc). HPLC: a linear gradient of 0–70% B was run in 30 min: main component 91%. (Mobile phase: A = MeOH/H_2O = 25:75, B = MeOH/H_2O = 80:20, by volume, both made with the addition of 0.05 M tetramethylammonium hydroxide (TMAH) and phosphoric acid till pH 3.0).

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, α-MSH

Since α-MSH was synthesized a long time ago, we re-purified this 13-peptide by chromatography on silica (Merck Fertigsäule) using solvent system c. TLC gave an R_f value of 0.32 in system d.
$[\alpha]_D^{21}$ −60.3° (*c*0.204, 10% HOAc). Lit.[7]: $[\alpha]_D^{23}$ −57.8°; −58.5° (ref. 12). The material was found to have the correct amino acid composition: peptide content 85.0%. LAO digestion: no racemization of any importance was detected. HPLC: a linear gradient of 0–70% B was run in 30 min; main component 97.0%.

Discussion

The dodecapeptide H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH, γ-MSH, was synthesized by fragment couplings in solution. The *N*-terminal pentapeptide and the *C*-terminal heptapeptide were purified before use, and gave the correct analysis. An azide reaction between these two peptides provided the dodecapeptide **11** which was deprotected and purified to give γ-MSH. The peptide showed the correct amino acid analysis and was free of racemized amino acid residues. The HPLC recording (making use of a critical system[8,17]) showed some small peaks in addition to the main component.
γ-MSH possessed only 2.5×10^{-4} of the activity of α-MSH in the melanotropic assay using lizards. γ-MSH is about as active as ACTH-(4–10) or α-MSH-(4–10)[18] viz. H-Met-Glu-His-Phe-Arg-Trp-Gly-OH, indicating that the *N*- and *C*-terminal parts of γ-MSH do not contribute significantly to its melanocyte-stimulating activity; this is contrary to the "head" and "tail" in α-MSH[19].
Recently, *Ling* et al.[7] published the synthesis by solid-phase methods of γ-MSH and three related peptides. The melanocyte-stimulating activities were determined using isolated skins of *Rana pipiens* as described by *Shizume* et al.[20]. Their γ_2-MSH, the same sequence that we have synthesized had only 1.4×10^{-4} (molar basis) the activity of α-MSH and

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was comparable to our peptide in the *Anolis Carolinensis* assay.
When tested for behavioural activity in the pole-jumping test[21] on extinction of conditioned avoidance response in rats, no increase of potency was found when compared with ACTH-(4-10) as a reference. Instead a new spectrum of central activities was found[6].

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Some stable substituted cycloheptatrienyl carbanions

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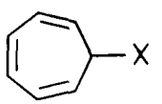
Abstract. The reaction of some 7-substituted cycloheptatrienes with KNH_2 in liquid ammonia is described. In several cases stable carbanions are formed. Their NMR spectra are consistent with a non-planar pentadienyl-like structure. A comparison of the ^{1}H-NMR spectra of **10** and **11** shows the paratropic character of the cycloheptatrienyl anions.

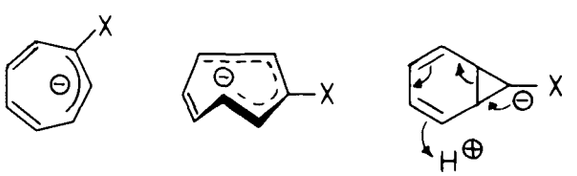
In the 1890's *Büchner* and coworkers reported various thermal isomerizations of ethyl esters of cycloheptatriene carboxylic acids[1]. These observations were subsequently interpreted as 1,5 – now called sigmatropic – shifts of hydrogen[2]. There remained one puzzling observation of *Büchner* in this area, namely that basic hydrolysis of the 7-ester (or 7-carboxamide) gave the 2-carboxylic acid[3].
Nozoe et al. investigated the action of base on 7-carboxy- (**1**), 7-carbamoyl- (**2**), 7-cyano- (**3**) and 7-phenylcycloheptatriene (**4**)[4]. In the first case **1** there is clearcut evidence for the successive formation of the 2-, 3- and 1-isomeric acids. In cases **2** and **3** the formation of the 2-isomer was observed, but any further isomerization was precluded by hydrolysis of the substituent. In case **4** the successive formation of these isomers is not clearly evident from the data.
When **1** and **3** were treated with sodium deuteroxide, exchange of the proton at C(7) preceded the isomerization to the 2-substituted cycloheptatriene. Closely related to this is the observation that reaction of **3** in THF with *n*-C_4H_9Li, followed by quenching with D_2O, yields 7-CN,7-D-cycloheptatriene[5].
Also of interest are several related reactions recently reported in the literature[6,7,8].



1–4, 8, 9, 12–19

1 X = CO_2H	**13** X = $CON(CH_3)C_6H_5$
2 X = $CONH_2$	**14** X = $CON(CH_3)_2$
3 X = CN	**15** X = $SO_2C_6H_5$
4 X = C_6H_5	**16** X = $SO_2C_6H_4Cl$-*m*
8 X = $SO_2C_2H_5$	**17** X = $SO_2C_6H_4Cl$-*p*
9 X = CO_2CH_3	**18** X = $SO_2C_6H_4OCH_3$-*m*
12 X = $CO_2C_2H_5$	**19** X = $SO_2C_6H_4OCH_3$-*p*

Nozoe et al. proposed allylic carbanions with a three-carbon coplanar structure (**5**) as intermediates for the base-catalysed 1,3-shifts of the proton. *Brown*, reviewing *Nozoe*'s work, briefly suggested a non-planar carbanion (such as **6**), which might be considered to be pentadienylic[9]. This suggestion has since gained in strength from the NMR spectrum of the benzocycloheptatrienyl anion, described by *Staley* and *Orvedal*[10], which points to a symmetrical structure (*cf.* also the remark of *Dewar*[11] about the unsubstituted cycloheptatrienyl anion). A further possibility that might be considered is a norcaradiene structure **7** which reacts with a proton as indicated.



5 **6** **7**

In this paper we describe our attempts to observe anions of simply substituted cycloheptatrienes by NMR spectroscopy. To that end several 7-substituted cycloheptatrienes were added to a slight excess of potassium amide in liquid ammonia at −60°C to −40°C and the spectra immediately

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