41. Synthesis, Opiate Receptor Binding and Analgesic Activity of Enkephalin Analogues

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Summary

The synthesis and biological testing of analogues of Met-enkephalin, a recently discovered opioid peptide from mammalian brain, are described. Testing involved determination of affinity constants for an opiate receptor site and of analgesic potency in the tail-flick test in mouse. The effects on opioid activity of modifying various parts of the enkephalin molecule are discussed. Tyr-D-Ala-Gly-MePhe-Met(O)-ol[1]) (FK 33-824), which was highly active in these tests, was subsequently selected for clinical testing.

The use of two complementary models – *in vitro* binding studies and *in vivo* test for analgesia – for the assessment of biological activity in the evaluation of analogues is explained.

1. Introduction. – Three laboratories announced independently in 1973 the successful identification of stereospecific opiate binding sites in brain tissue [1–3]; subsequently opiate receptors were found in the brains of all vertebrate species examined [4]. The existence of an endogenous ligand for the opiate receptor was confirmed by the demonstration of a morphine-like factor in pig [5], rat [6], calf [7], and human brain [8]. *Hughes et al.* [9] characterized in 1975 the factor from pig brain as a mixture of two pentapeptides, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, which they named Met- and Leu-enkephalin respectively. The same two peptides, though in different proportion, were subsequently identified in calf brain [10]. The hypothesis of these two pentapeptides having a role as inhibitory neurotransmitters in the central nervous system [11] [12] is receiving increasing support [13] [14].

The amino acid sequence of Met-enkephalin is identical with the sequence 61 to 65 of β-lipotropin [9]. Other morphinomimetic fragments of β-lipotropin, called α-, β-, and γ-endorphin and possessing 16, 31, and 17 amino acid residues respectively, have the same amino-terminal sequence as Met-enkephalin. These recent advances in neuroscience research have been expertly reviewed by several authors [12] [15–18].

Met- and Leu-enkephalin elicit weak and short-lived analgesia when administered into the brain ventricles of mice [19] and rats [20]. Although the short duration of action of these peptides is in keeping with their postulated role

[1]) The ending -ol added to the symbol of an amino acid designates the aminoalcohol obtained by reduction of the α-carboxyl group of the amino acid.

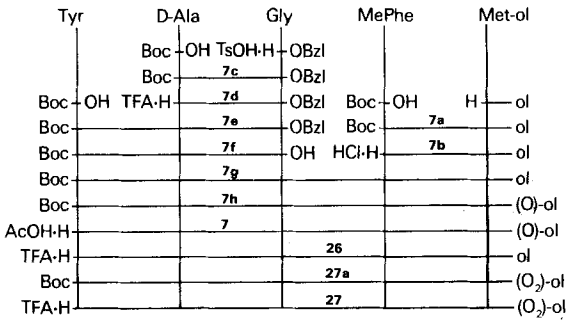
as neurotransmitters, their rapid inactivation *in vivo* and *in vitro* [21–24] has hampered detailed investigation of their pharmacological properties. We therefore endeavoured to synthesize more stable and more potent analogues of the naturally occurring enkephalins, using as our evaluation criteria the affinity of the analogues for the opiate receptor in rat brain tissue *in vitro* [25] and their analgesic potency in the tail-flick test in mouse [26].

Information gained from both of these tests has resulted in the rapid development of promising analogues, one of which, coded FK 33-824, was found to have potent and long-lasting analgesic properties in several species of laboratory animals [27] and a variety of effects in man [28].

We report here on design, synthesis and properties of the Met-enkephalin analogue FK 33-824 and discuss some aspects of structure activity relationship for a group of closely related derivatives.

2. Synthesis. – 2.1. *Synthesis of H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol* (**7**)[2]). The synthesis of **7** by classical solution methods took place according to *Scheme 1*[3]).

Scheme 1. *Synthesis of H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol* (**7**).



All peptide couplings in this synthesis were effected by the mixed carbonic acid anhydride method [29]. The Boc-group was used exclusively for the intermediate protection of amino groups. The carbinol group of methioninol and the phenolic hydroxyl of tyrosine remained unprotected throughout the synthesis. Fragments **7f** and **7b** were coupled by the mixed anhydride method to give the protected pentapeptidyl alcohol **7g**, which was purified by column chromatography on silica gel. In another synthesis (not reported in the Experimental Part) the coupling of fragments **7f** and **7b** with dicyclohexyl-carbodiimide (DCCI) and 1-hydroxy-benzotriazole (HOBt) [30] gave a somewhat more complex mixture of crude products, from which however column chromatography gave a similar yield of pure **7g**. Sulfide **7g** was rapidly oxidized by 1.1 equiv. of hydrogen peroxide in acetic acid solution to yield sulfoxide **7h** quantitatively.

A larger excess of hydrogen peroxide and much longer reaction times led to the corresponding protected sulfone **27a**. Final deprotection of the intermediate

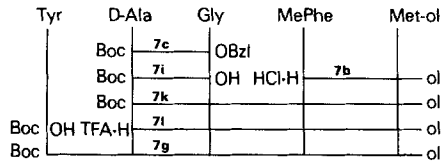
[2]) FK 33-824 in previous publications [27] [28].

[3]) Intermediates in the synthesis, *e.g.*, of analogue **7**, are referred to as **7a**, **7b** ... throughout this text.

sulfoxide **7h** with TFA/CH_2Cl_2 and anion exchange gave H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol acetate (**7**), which TLC. showed to be homogeneous. This route was preferred to the one involving the oxidation of H-Tyr-D-Ala-Gly-MePhe-Met-ol trifluoracetate (**26**) in acetic acid or in aqueous solution, which led to the same product **7** but in a lesser state of purity. H-Tyr-D-Ala-Gly-MePhe-Met(O_2)-ol trifluoroacetate (**27**) was similarly obtained from the protected sulfone **27a**. Thus sulfoxide **7** as well as the corresponding sulfide **26** and sulfone **27**, which were separable by TLC. and column chromatography, could be derived from the same precursor **7g**.

Alternatively, **7g** was synthesized by a [1 + 4] condensation as shown schematically in *Scheme 2*. Coupling Boc-L-tyrosine *via* its mixed anhydride to the tetrapeptidyl alcohol **7l** gave the protected pentapeptidyl alcohol **7g**, identical with the one obtained by the [3 + 2] approach.

Scheme 2. *Alternative Synthesis of Intermediate* **7g**.



The risk of partial racemization in the coupling steps was minimized by building up fragments stepwise with Boc-protected amino acids and by fragment coupling to the achiral glycine residues of tripeptide of **7f** or dipeptide **7i**. Furthermore, only weak bases, *e.g.*, *N*-methyl- or *N*-ethyl-morpholine, were used in equimolar amounts. Of the starting materials, Boc-*N*-methyl-L-phenyl-alanine was prepared according to a newer procedure which, of the various possible methods, has been shown to be the one that produces the least amount of racemized derivative [31]. L-Methioninol was obtained by racemization-free [32] reduction of methyl L-methioninate hydrochloride with $NaBH_4$ in ethanol.

Sulfoxides possess a chiral *S*-atom and the oxidation of sulfide **7g** by hydrogen peroxide was therefore expected to give a mixture of the diastereoisomeric sulfoxides **7h**. Stereoselective oxidation has been reported for a few substrates [33] [34]; however, ^{13}C-NMR. spectra of **7** indicated the presence of a mixture, approximately 1:1, of the sulfoxide isomers in this compound as well as in other (data not reported) analogues investigated. NMR. spectra of compound **7** (data given in the experimental Part) confirmed the correct structure of the final product, in particular of its *N*-methylphenylalanine and methioninol-sulfoxide residues, the former giving a very low colour value with ninhydrin and the latter going undetected in standard amino acid analysis of peptides [35].

The stability of Tyr-D-Ala-Gly-MePhe-Met(O)-ol (**7**) in the presence of proteolytic enzymes was assessed by incubating it with either human plasma or with leucine-aminopeptidase (LAP). Analogue **7** was unaffected when exposed to human plasma for 24 hours; by contrast, Met-enkephalin was almost completely hydrolyzed to its constituent amino acids in the same period. LAP split

about half the tyrosine from analogue **7** in 4 hours and all of it in 24 hours; Met-enkephalin, under the same conditions, lost all its tyrosine in 1 minute. These results show the greatly increased stability of analogue **7** against enzymatic degradation and concomitant loss of activity as compared to Met-enkephalin.

2.2. *Synthesis of Analogues of H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol* (**7**). Owing to the large number of analogues, the description of their synthesis in this section

Table 1. *Analogues of H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol.* Analgesic activity and inhibition of ^{3}H-Naloxone binding.

		$[\alpha]_D^{20}$ [a]	Salt [b]	IC_{50} [c]	Analgesia [d]
7	H-Tyr — D-Ala — Gly — MePhe — Met(O)-ol	− 7.0	ac	12 ± 0.8 (6)	+ + +
8	H-Arg — ol	− 0.5	ac	8.5 ± 0 (2)	+ +
9	H-Glu — ol	− 15.3	ch	130 ± 10 (2)	+ +
10	H-Phe — ol	− 13.6	ch	11 ± 2 (2)	+ + +
11	H-D-Phe — ol	− 45.5	ch	9000 ± 3000 (2)	+
12	Ac — ol	− 63.8	n	2100 ± 100 (2)	+
13	H-MeTyr — ol	− 15.9	ch	10 ± 2 (4)	+ + +
14	H-CbmTyr[f] — ol	− 17.4	ch	125 ± 24 (3)	+
15	Me_2Tyr — ol	− 3.7	tf	20 ± 7 (2)	+ +
16	H-Tyr(Me) — ol	− 19.6	ch	390 ± 10 (2)	+ + [g]
17	H-D-Tyr — ol	− 58.6	ch	3600 ± 600 (2)	0
18	D, L-Hyhp[h] — ol	− 39.5	n	[e]	0
19	H-Tyr-D-MeAla — ol	+ 2.8	ch	26 ± 6 (3)	+ +
20	H — D-Ser — ol	− 10.6	tf	150 ± 15 (3)	+ +
21	H — D-Met — ol	− 4.8	tf	33 ± 5 (3)	+ +
22	H — D-Met(O) — ol	− 11.1	tf	20 ± 4 (2)	+ +
23	H — D-Ala — Phe(*p*-Cl) — ol	+ 33.5	tf	41 ± 1 (2)	+ +
24	H — Phe(*p*-NO_2) — ol	+ 33.7	ch	9.0 ± 2 (2)	+ + +
25	H — MePhe(*p*-NO_2) — ol	− 11.6	tf	5.4 ± 1.6 (4)	+ +
26	H — MePhe — Met-ol	− 4.8	tf	39 ± 6 (2)	+ +
27	H — Met(O_2)-ol	− 4.6	tf	11 ± 1.2 (2)	+ + +
28	H — D-Met(O)-ol	− 14.9	tf	110 ± 10 (2)	+ +
29	H — S-Me-Cys(O)-ol	− 42.8	ch	115 ± 13 (3)	+ +
30	H — Leu-ol	− 7.5	tf	85 ± 5 (2)	+ + [i]
31	H — Pro-ol	+ 4.0	tf	140 ± 49 (4)	+ + [i]

[a] Optical rotation in 95% acetic acid, concentration corrected for peptide content, $c = 1$ of free base.

[b] ac, acetate; b, free base; ch, hydrochloride; n, neutral or inner salt; tf, trifluoroacetate.

[c] Radioreceptor assay. IC_{50}, concentration (10^9 mol · l^{-1}) of analogue (free base) required to inhibit specific binding of [^{3}H]-naloxone (1 nM) to pre-incubated membranes from rat brain (minus cerebellum) by 50%. Values ± s.e.m.; number of experiments in parentheses. For details of the procedure see Experimental Part.

[d] Analgesia as measured in the tail-flick test of the mouse 30 min after *i.v.* administration; + + + = analgesia observed at doses below 1.8 mg · kg^{-1}; + + = analgesia observed at doses 1.8–18 mg · kg^{-1}; + = analgesia observed at doses 18–180 mg · kg^{-1}; 0 = no analgesia observed at doses below 180 mg · kg^{-1}.

[e] Concentration required higher than 10^{-5} mol · l^{-1}.

[f] CbmTyr, *N*-cyclobutylmethyl-L-tyrosine.

[g] Analgesia measured after 120 minutes.

[h] D,L-Hyhp, 2-hydroxy-3-(*p*-hydroxyphenyl)-propionic acid.

[i] Analgesia measured after 5 minutes.

is limited to the main features of each group, concentrating on the differences from the synthesis of analogue **7**. Details are given in the Experimental Part for new starting materials only. Specific rotations are given in *Table 1*. The structures of new compounds were corroborated by amino acid analysis, NMR. spectroscopy and, for some analogues, by field desorption mass spectrometry. The homogeneity of the compounds was determined by TLC. and in some cases also by HPLC. Methods of protection, coupling, deprotection and purification were generally the same as those already described for compound **7** in the preceding section.

The hexapeptidyl alcohols **8**, **9**, **10** and **11** were prepared by coupling the respective *N*-protected amino acids by way of their active esters, *e.g.* with *p*-nitrophenol, to analogue **7**. The sidechain of glutamic acid was esterified with *t*-butyl alcohol, which was subsequently removed, together with Boc, in the final step to **9**. The guanidino side-chain of arginine was protected by two Cbo-groups, which were finally removed by treatment with BTFA [36] [37] to give **8**. Acetylation of **7** with acetic acid pentachlorophenylester led directly to **12**.

Coupling 2-hydroxy-3-(*p*-hydroxyphenyl)-propionic acid (D,L-Hyhp) or the respective Boc-protected tyrosine derivatives with the tetrapeptidyl alcohol **7l** by the mixed anhydride method led, after the usual steps, to analogues **13**, **16**, **17** and **18**. The *N*-cyclobutylmethyl derivative **14** was obtained by reductive alkylation of **7** with cyclobutane carbaldehyde and $NaBH_3CN$ in methanol; in a similar reaction of **7** with an excess of formaldehyde in acetonitrile the *N*-dimethyl derivative **15** was formed.

Replacing Boc-D-alanine in the synthesis of fragment **7f** by Boc-*N*-methyl-D-alanine or Boc-D-serine and coupling the resulting tripeptides with fragment **7b** gave, as in the synthesis of **7**, analogues **19** and **20** respectively. Hydrogenolysis, as used in the synthesis of **7f**, could not be used for the synthesis of the corresponding tripeptides containing D-methionine or D-methionine-sulfoxide in the preparation of analogues **21** and **22**. Therefore, these tripeptides were synthesized from glycine by stepwise elongation with the appropriate Boc-amino acids by way of their *p*-nitrophenylesters. Coupling of Boc-Tyr-D-Met-Gly-OH with fragment **7b** followed by oxidation to the di-sulfoxide and deprotection as in the synthesis of **7**, gave analogue **22**. In order to obtain the selectively oxidized analogue **21**, Boc-Tyr-D-Met-Gly-OH was coupled with HCl · MePhe-Met(O)-ol and the product treated with TFA.

Replacing Boc-*N*-methyl-phenylalanine in the preparation of fragment **7b** by Boc-*p*-chloro-L-phenylalanine, Boc-*p*-nitro-L-phenylalanine or Boc-*N*-methyl-*p*-nitro-L-phenylalanine (**25b**) and coupling the resulting dipeptidyl alcohols with fragment **7f**, as in the synthesis of **7**, gave analogues **23**, **24** and **25** respectively.

Replacing L-methioninol in the preparation of fragment **7b** by D-methioninol or by *S*-methyl-L-cysteinol (**29a**) and coupling with fragment **7f** as before gave, after oxidation and deprotection, analogues **28** and **29** respectively. Similarly, replacing L-methioninol in fragment **7b** by L-leucinol or L-prolinol and coupling with fragment **7f** followed by deprotection, led directly to analogues **30** and **31** respectively.

3. Results and Discussion. – The essential role of the tyramine moiety, $(p)HO-C_6H_4-CH_2-CH(NH_2)-$, for the opiate receptor binding and analgesic activity of the enkephalins was soon recognized as was the occurrence of this structural element in a large number of known opiates [38]. It has also been demonstrated that inactivation of the natural enkephalins by serum or tissue proceeds by enzymatic degradation starting with the splitting of the Tyr-Gly peptide bond [21] [23], producing fragments devoid of any opiate receptor binding and analgesic activity. Our first objective was therefore the stabilization of this *N*-terminal peptide bond.

Replacing L-tyrosine in Met-enkephalin (**1**) by its D-antipode gave analogue **2**, which showed no affinity for the opiate receptor and only transient analgesia

Table 2. *Analogues of Met-Enkephalin.* Analgesic activity and inhibition of ^{3}H-Naloxone binding.

		$[\alpha]_D^{20}$ [a]	Salt [b]	IC_{50} [c]	Analgesia [d]
1	H-Tyr — Gly — Gly — Phe — Met-OH	+26.1	n	412±69 (5)	+[f]
2	H-D-Tyr — OH	+25.1	ac	[e]	0[f]
3	H-Tyr — D-Ala — OH	+39.4	tf	61± 7 (3)	+[f]
4	H — Gly — Met-ol	+26.4	b	50±13 (3)	+[f]
5	H — D-Ala — Met-ol	+39.1	b	20± 3 (4)	+
6	H — Met(O)-ol	+32.8	ch	60± 7 (3)	++
7	H-Tyr — D-Ala — Gly — MePhe — Met(O)-ol	− 7.0	ac	12± 0.8 (6)	+++

[a–e]) See notes to *Table 1.* [f]) Analgesia measured 15 sec after *i.v.* administration.

in the mouse after *i.v.* administration. More promising was the replacement of 2-glycine by D-alanine (→**3**), a modification which has previously been successfully applied to several other peptide hormones. The carboxyl group of methionine was also replaced by a carbinol group ($-CH_2OH$) (→**4**) in order to stabilize the *C*-terminal against attack by carboxypeptidases. Both these modifications increased receptor-binding and produced analgesic activity after intravenous administration. Combination of these two modification in the same molecule gave analogue **5**, which caused analgesia lasting more than 30 minutes after intravenous and subcutaneous administration in the mouse, thus demonstrating the greatly increased stability of this compound *in vivo.*

Attempts were then made to improve the gastro-intestinal absorption of analogue **5** and its penetration of the blood-brain barrier by the oxidation of methioninol to its sulfoxide (→**6**) and the addition of a methyl group to the nitrogen of the phenylalanine residue, giving [2-D-alanine, 4-*N*-methyl-phenylalanine, 5-methioninol-sulfoxide] enkephalin (**7**).

Compound **7** exhibited very high affinity for the opiate receptor as shown in *Table 2*, and it had the most potent analgesic properties. In a more detailed pharmacological study [27] it was shown that, on a molar basis, **7** was 30,000 times more potent than Met-enkephalin and 1,000 times more potent than morphine after administration directly into the brain ventricles of mice. Besides showing analgesic properties after parenteral and oral administration, analogue **7** had a duration of action exceeding that of morphine, probably as a result of its resistance to proteolytic enzymes (see Section 2.1). The high potency and enhanced stability of **7** made this compound a suitable base from which to investigate structure/activity relationships among the enkephalins. Several other studies [39–44] have covered a wide range of modifications of the enkephalin molecule, reporting on affinity for opiate receptors, activity on the guinea-pig ileum preparation *in vitro* and analgesic activity in some animal models. However, none of the analogues described in these studies has shown the high analgesic potency and the long duration of action of **7**.

In *Table 1* affinity for an opiate receptor preparation and analgesic activity as assessed in the tail flick test in mouse are given for a series of analogues, each differing from **7** by the substitution of a single amino acid or amino alcohol residue. Affinity is expressed as IC_{50}, a low IC_{50} value indicating a high

affinity for the opiate receptor. Analgesic activity is expressed on a 4-point rating scale, the highest score (+++) being attained by compounds which are at least as potent as morphine[4]).

The importance of the terminal amino group in determining activity was studied in a number of analogues, **8–15** and **18**. When this group was replaced by the isosteric but non ionic hydroxyl group the resulting analogue **18** had no affinity for the opiate receptor at all and was inactive in the tail flick test. Activity was maintained when the amino group was substituted with a single methyl group, as in analogue **13**, whereas two such methyl groups depressed affinity and reduced analgesic activity, as in analogue **15**. Reduced receptor binding and analgesic activity was also observed with one bulky substituent, cyclobutylmethyl, in analogue **14**. Analogue **17** provided evidence for the high stereoselectivity of the ligand-receptor interaction, by demonstrating the absolute need for the L-configuration of tyrosine[5]) in position 1. Acetylation of the amino group gave analogue **12**, which showed weak analgesic activity and had a low affinity for the opiate receptor. A more complex picture was observed when the tyrosine amino group was acylated with various amino acids to give the hexapeptidyl alcohols **8–11**. Addition of a basic (→**8**), acidic (→**9**), or neutral (→**10**) L-amino acid did not appreciably diminish analgesic activity, whereas when a D-amino acid (→**11**) was added to analogue **7** this property was greatly reduced. We interprete the differing *in vivo* effect of the acyl groups on analgesic activity as being due to enzymatic cleavage of an L-amino acid – but not a D-amino acid or acetyl group – to give analogue **7**. The results obtained in the binding assay correlate well with the analgesic potency of analogues **8**, **10** and **11**. It is concluded, therefore, that with the exception of glutamic acid in analogue **9**, splitting of L-amino acids bound to tyrosine occurred during the incubation period of these analogues with the rat brain preparation. In support of this conclusion the activity of an enzyme with the properties of a L-aminopeptidase has been held responsible for the rapid inactivation of natural Met- and Leu-enkephalin in particulate fractions of rat brain homogenate [21].

Methylation of the phenolic hydroxyl group of tyrosine gave analogue **16**, which showed analgesic activity in the tail flick test but only after a delay of about 60 minutes. This may be due to slow biotransformation of **16** to **7** *in vivo*. The same transformation probably does not take place under the *in vitro* conditions of the binding assay, which showed the affinity of the *O*-methyl derivative **16** for the opiate receptor to be at least 30 times lower. This compares with a reduction in affinity by a factor of more than two thousand in the morphine series, where *O*-methylation gives codeine [25].

Substitution onto D-alanine in position 2 gave analogues **19–22**, all of which showed reduced affinity for the opiate receptor and a slightly weaker analgesic effect.

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[4]) Morphine $IC_{50} = 73$ nM; $ED_{50} = 1.8$ mg/kg^{-1} [27].

[5]) The affinity of pure analogue **17** may be even lower than the value of 3600 nM given in *Table 2* since contamination of the sample by a very small amount, *e.g.* 0.3%, of the L-tyrosine isomer cannot be ruled out and could have been responsible for the low, but still significant, binding of our preparation.

The addition of a nitro group to the *N*-methyl-phenylalanine residue gave **25**, which exhibited increased binding to the opiate receptor, but slightly reduced analgesic activity. The *p*-chlorophenylalanine derivative **23** had an affinity of only about one third that of **7** and was less potent than the *p*-nitro-phenylalanine derivative **24**, which was equipotent in the binding and analgesia tests with **7**.

Of the analogues formed by substitution for the *C*-terminal methioninolsulfoxide, only the sulfone **27** was found to have activity comparable to **7**. Analogues obtained with L-methioninol (**26**), D-methioninol sulfoxide (**28**) and the homologous *S*-methyl-L-cysteinol sulfoxide (**29**) had a lower affinity for the opiate receptor and were less potent analgesics. All the analogues containing sulfur had a remarkable long duration of action – up to 7 hours with the 18 $mg \cdot kg^{-1}$ dose – whereas analgesic activity in the case of the sulfur-free analogues with L-leucinol (**30**) or L-prolinol (**31**) was detectable only during the first thirty minutes after administration.

Evaluation of the analogues with the greatest analgesic potency (marked +++ in *Table 1*) on a more quantitative basis revealed that only analogue **13**, with *N*-methyl-tyrosine, was more potent than **7**. Indeed, it was about 5 times more potent than **7** after subcutaneous ($ED_{50} = 0.3$ $mg \cdot kg^{-1}$) and oral ($ED_{50} = 20.5$ $mg \cdot kg^{-1}$) administration in the tail flick test in mouse.

4. Conclusion. – Structural modification of natural Met-enkephalin has given analogues with higher affinity for the opiate receptor and greater analgesic potency than the parent compound. Analogue **7**, coded FK 33-824, was found to be a particularly potent analgesic after parenteral and, surprisingly, even after oral administration. Its pharmacological effects last for several hours, probably as a consequence of its resistance to enzymatic degradation. FK 33-824 is currently undergoing clinical testing [28].

Further modification of the molecule produced analogues with predominantly weaker analgesic potency. A few however, were found to have comparable potency to FK 33-824 and only one, the 1-*N*-methyl-tyrosine analogue **13**, was more potent.

This study illustrates the advantages of a method for the assessment of the biological activity of analogues of a natural compound – in this case Met-enkephalin – based on two complementary models. The *in vitro* test, which uses highly specific receptor preparations taken from the target organ (rat brain in this study), is designed to identify all substances which interact with the receptor, regardless of the pharmacological activity triggered by this interaction in the intact cell or organ; it reveals the relevant structural features of the enkephalins, *i.e.*, essential groups, minimal sequence and stereospecificity, without interference from the transport, distribution and, to some extent, degradation phenomena which are an inevitable part of *in vivo* tests. At the same time, a rapid and relevant *in vivo* test to detect primary pharmacological activity (tail flick test for analgesia in this study) represents a screen for useful compounds, *i.e.*, analogues that are effective after administration by one of the preferred routes and have the profile of activity needed for potential new drugs.

We should like to thank *H. R. Loosli* for the interpretation of the NMR. spectra.

Experimental Part

Materials. - [^{3}H]-Naloxone, specific radioactivity 17 Ci mmol^{-1}, was obtained from *New England Nuclear Corporation.* Leucine-aminopeptidase, prepared from bovine lens, was obtained from *Serva.* Amino acid derivatives were obtained from commercial sources or prepared by published procedures and checked by TLC. and optical rotation before use. Other chemicals and solvents were of reagent grade or analytical grade quality and used without purification.

Methods. - Elemental analysis was performed in our microanalytical laboratory *(W. Pfirter).* For amino acid analysis, peptides were hydrolyzed with 6N HCl in sealed, evacuated vials at 115° for 16 h and analyzed according to *Stein & Moore* with a *Biotronik,* LC 6000 apparatus. Optical rotations were measured with a *Perkin-Elmer* 241 polarimeter. ^{1}H-NMR. spectra at 90 MHz with 3-trimethylsilyl-propionic acid as internal reference ($\delta = 0$ ppm) and ^{13}C-NMR. at 22.63 MHz with dioxane as internal reference ($\delta = 67.8$ ppm) were recorded in D_2O on a *Bruker* HX-90-E spectrometer. High performance liquid chromatography (HPLC.) was performed on columns 4.6 × 250 mm of RP 18 on LiCHrosorb 5 µ *Merck* and eluted with gradients of acetonitrile and 0.01M ammonium acetate, pH = 5. Thin layer chromatography (TLC.) was performed on precoated plates of silica gel HF_{254} *Merck* with the following solvent systems: A = methylene chloride/methanol 4:1, B = methylene chloride/methanol 7:3, C = methylene chloride/methanol/water 14:6:1, D = butanol/acetic acid/water 4:1:2, E = acetone/acetic acid/methanol 8:1:1. Plates were examined under UV_{254} light and sprayed with ninhydrin, chlorine-TDM [46], or *Folin*-reagent.

Binding assay with [^{3}H]-naloxone. - Male rats (OFA 120–200 g) were decapitated and their brains minus cerebella were rapidly removed, placed in 30 volumes *(v/w)* of ice-cold 50 mM Tris/HCl buffer, pH 7.4 at 37°, and homogenized with a *Polytron* PT-20 ST for 30 sec. at a setting of 7 (full scale 10). The homogenate was centrifuged at 48,000 × g for 10 min at 4°, the pellet was resuspended (*Polytron,* 30 sec.) in 30 volumes of the same buffer, pre-incubated for 30 min at 37° and recentrifuged. The pellet was resuspended (*Polytron,* 30 sec.) in 100 volumes of 50 mM Tris/HCl buffer, pH 7.4 at 25°; this buffer was used in all subsequent steps. The assay, in a glass test tube (16 × 100 mm), contained 1.7 ml of tissue suspension (corresponding to 17 mg of wet brain), 100 µl of the drug (peptide analogue) solution in buffer, 100 µl of 2M NaCl in buffer (100 mM final concentration), and 100 µl of 20 nM [^{3}H]-naloxone (1 nM final concentration). The tubes, prepared in triplicate, were incubated at 25° for 40 min. The incubation was terminated by rapid filtration under suction through *Whatman* GF/B glass fiber filters at 20°. The filters were washed twice with ice-cold buffer (5 ml) and transferred to scintillation vials containing 10 ml of *Insta-Gel (Packard Instrument Company)* scintillation fluor. Vials were shaken mechanically for 4 h at room temp. and counted in a liquid scintillation spectrometer with 42% efficiency. Specific [^{3}H]-naloxone binding was defined as the difference between the binding of [^{3}H]-naloxone in the presence and absence of 1 µM levallorphan; it accounted for approximately 85% of the total binding. IC_{50} was defined as the concentration of drug required to inhibit by 50% the specific [^{3}H]-naloxone binding; it was calculated by log-probit analysis of four to six drug concentrations. Values of IC_{50} of enkephalin analogues given in *Tables 1* and *2* are the means of at least two independent determinations using freshly prepared tissue and drug solutions.

The tail flick test for analgesics in the mouse. - The analgesic effects of the test compounds were determined in the tail flick test [26]. Groups of 5, 8, or 10 unstarved male and female mice (*Sandoz* strain OFI) each weighing 16 to 25 g were used. Thirty minutes before administration of the test compound each mouse was put in an individual perspex cylinder constructed such that it could not turn around or move forward and so that its tail protruded and lay along a narrow groove. A fixed point of the tail (about 35 mm from the root) of each mouse was exposed in turn to a beam of radiant heat from a lamp placed directly under the tail. The time in sec. that the mouse took to flick its tail out of the beam was recorded twice, once 30 min and once 15 min before administration of the test compound. A mouse was only used if the reaction times differed by less than 25%. The mean pre-administration reaction time for each mouse (5–8 sec) was then determined. The test compound was administered by the preselected route in physiological saline solution and the reaction times determined at fixed times after administration. These times were chosen such as to allow the duration of action of the test compounds as well as their potency to be established. An extension of the reaction time after administration of the test compound of more than 75% compared to the

mean pre-administration reaction time for that particular mouse was regarded as an analgesic response. The number of animals exhibiting analgesia at each dose and time interval was recorded.

The ED_{50} (95% confidence limits) of each test compounds, estimated according to the method of *Lichtfield* [47], was taken to be the dose that prolonged the mean pretreatment reaction time by more than 75% in 50% of the animals (minimum of 8 mice per dose, 3 doses per drug).

Enzymatic digestion. – a) Leucine-aminopeptidase, 0.2 mg in 0.1 ml of buffer, was added to a solution of the peptide (0.8 µmol) and leucine (1 µmol) in 1.3 ml of a buffer, 0.14M in triethylamine, 0.002M in $MgCl_2$, adjusted to pH 8.5 with acetic acid and incubated at 37°. Samples were removed after 1 min and after 24 h, quenched by the addition of 5 volumes of 1N CH_3COOH and analyzed for free amino acids using leucine as internal standard.

Met-enkephalin	Tyr 1.00 (0.92) Gly n.d. (1.71) Phe n.d. (0.87) Met n.d. (0.96)
Analogue 7	Tyr 0.01 (1.00) no other amino acids detected
	Tyr 0.45 after 4 h

(Values after 24 h in parenthesis. n.d. = not detected, *i.e.* less than 0.01)

b) Human plasma, 0.1 ml, was added to a solution of the peptide (0.8 µmol) and leucine (1 µmol) in 1.3 ml of 0.05M *Tris* buffer, pH 7.4 and incubated at 37°. A sample was removed after 24 h, quenched by the addition of 5 volumes of 1N CH_3COOH and analyzed for free amino acids using leucine as internal standard. Values were corrected for the background of amino acids in a human plasma blank.

Met-enkephalin	Tyr 0.87 Gly 1.05 Phe 0.75 Met 0.85[a])
Analogue 7	Tyr 0.04 no other amino acids detected

[a]) Additional peak, probably glycyl-glycine.

The presence of unchanged analogue **7** in the samples was confirmed by HPLC.

Synthesis. – *Boc-MePhe-Met-ol* (**7a**). Boc-MePhe-OH [48] (3.1 g, 11 mmol) and *N*-ethyl-morpholine (1.4 ml, 11 mmol) were dissolved in tetrahydrofuran (30 ml) and cooled to −20°. Isobutyl chloroformate (1.45 ml, 11 mmol) was added followed, after stirring at −15° for 10 min, by a cold solution of L-methioninol [49] (1.8 g, 13.3 mmol) in THF (10 ml). After stirring at −5° for 3 h and at 20° for 1 h, the mixture was diluted with ethyl acetate (170 ml) and ether (170 ml) and extracted with several portions of water, 2M citric acid, 1M $KHCO_3$ and 30% NaCl-solution. The organic layer was dried over anhydrous Na_2SO_4 and the solvents removed *in vacuo* to leave **7a** as a viscous liquid: 3.8 g (86%), $[\alpha]_D^{20} = -49.3°$ ($c = 1$, DMF).

$C_{20}H_{32}N_2O_4S$	Calc.	C 60.6	H 8.1	N 7.1	O 16.1	S 8.1%
(396.6)	Found	„ 60.5	„ 8.4	„ 6.8	„ 16.5	„ 8.5%

H-MePhe-Met-ol, HCl (**7b**). **7a** (4 g, 10 mmol) and methyl ethyl sulfide (3 ml, 33 mmol) were dissolved in dioxane (5 ml), cooled to 0° and 3.6N HCl in dioxane (20 ml) added. After 2 h ether was added to complete crystallization and the product, **7b**, isolated by filtration: 2.9 g (87%). It was recrystallized from ethanol/ether: 2.0 g (60%); m.p. 154° (dec.), $[\alpha]_D^{20} = +12{,}6°$ ($c = 1$, methanol).

$C_{15}H_{25}ClN_2O_2S$	Calc.	C 54.1	H 7.6	Cl 10.7	N 8.4	O 9.6	S 9.6%
(332.89)	Found	„ 54.1	„ 7.8	„ 10.7	„ 8.5	„ 9.8	„ 9.7%

Boc-D-Ala-Gly-OBzl (**7c**). Boc-D-Ala-OH [50] (2.3 g, 12 mmol) and *N*-methyl-morpholine (1.3 ml, 12 mmol) were dissolved in THF (20 ml) and cooled to −20°. Isobutyl chloroformate (1.6 ml, 12 mmol) was added followed, after stirring at −15° for 5 min, by a cold solution of H-Gly-OBzl, Ts-OH [51] (4 g, 12 mmol) and *N*-methyl-morpholine (1.6 ml, 12 mmol) in DMF (10 ml). After stirring at −15° for 2.5 h the mixture was diluted with ethyl acetate (250 ml) and work-up continued as in the preparation of **7a**. The crude product, obtained after evaporation of the solvents, was recrystallized from ethyl acetate/ether/petroleum ether (b.p. 30–60°): 3.4 g (85%), m.p.. 84°, $[\alpha]_D^{20} = +11.3°$ ($c = 1$, DMF).

$C_{17}H_{24}N_2O_5$	Calc.	C 60.7	H 7.2	N 8.3	O 23.8%
(336.39)	Found	„ 60.9	„ 7.3	„ 8.4	„ 23.6%

Boc-Tyr-D-Ala-Gly-OBzl (**7e**). **7c** (15 g, 44 mmol) was dissolved in a mixture of trifluoroacetic acid (135 ml) and methylene chloride (15 ml). After 45 min at 20°, the solvents were removed *in vacuo*, the evaporation repeated with the addition of toluene and the residue dried *in vacuo* over KOH-pellets to give **7d** as an amorphous solid. This was dissolved in THF (150 ml), cooled to −15° and *N*-ethyl-morpholine (5.8 ml, 46 mmol) added (Solution A).

Boc-Tyr-OH (12.4 g, 44 mmol) and *N*-ethyl-morpholine (5.6 ml, 44 mmol) were dissolved in THF (150 ml) and cooled to −20°. Isobutyl chloroformate (5.8 ml, 44 mmol) was added followed, after stirring at −15° for 5 min, by Solution A. After stirring at −15° for 2 h and at 5° for 15 h the mixture was concentrated *in vacuo* to about one half its volume, diluted with ethyl acetate (1.5 l) and work-up continued as before. The sirupy residue, obtained after evaporation of the solvents, was triturated with methylene chloride/ether/hexanes and dried *in vacuo* to give the product **7e** as a foamy residue: 19 g (86%). $[\alpha]_D^{20} = +25.6°$ ($c = 1$, CH_3COOH 95%).

$C_{26}H_{33}N_3O_7$	Calc.	C 62.5	H 6.7	N 8.4	O 22.4%
(499.56)	Found	„ 62.2	„ 7.0	„ 8.0	„ 22.2%

Boc-Tyr-D-Ala-Gly-OH (**7f**). **7e** (12 g, 24 mmol) was dissolved in methanol (140 ml), 10% Pd/C (1.2 g) was added and the mixture hydrogenated at atmospheric pressure at 20° for 3 h. The catalyst was removed by filtration through a bed of *Celite*, the filtrate concentrated *in vacuo* and the product **7f** precipitated with ether: 8.8 g (86%); m.p. 166° (dec.), $[\alpha]_D^{20} = +41.6°$ ($c = 1$, methanol).

$C_{19}H_{27}N_3O_7$	Calc.	C 55.7	H 6.6	N 10.3	O 27.4%
(409.44)	Found	„ 55.6	„ 6.1	„ 10.0	„ 27.6%

Boc-Tyr-D-Ala-Gly-MePhe-Met-ol (**7g**). a) **7f** (1.23 g, 3 mmol) and *N*-ethyl-morpholine (0.38 ml, 3 mmol) were dissolved in THF (30 ml) and cooled to −20°. Isobutyl chloroformate (0.39 ml, 3 mmol) was added followed, after stirring at −15° for 10 min, by a cold solution of **7b** (1.16 g, 3.3 mmol) and *N*-ethyl-morpholine (0.45 ml, 3.6 mmol) in THF (15 ml). After stirring at −15° for 1 h and at −5° for 20 h the mixture was diluted with ethyl acetate (450 ml) and work-up continued as before. The crude product obtained after evaporation of the solvent was purified by column chromatography on silica gel 0.06–0.2 mm *Merck* and eluted with methylene chloride/methanol/acetic acid 100:10:1. Fractions were checked by TLC., pooled and evaporated: 1.1 g (53%). $[\alpha]_D^{20} = -28.2°$ ($c = 0.98$, methanol). Rf 0.79 (B), 0.63 (A).

b) Boc-Tyr-OH (0.28 g, 1 mmol) and *N*-methyl-morpholine (0.11 ml, 1 mmol) were dissolved in THF (5 ml) and cooled to −20°. Isobutyl chloroformate (0.12 ml, 1 mmol) was added followed, after stirring at −20° for 5 min, by a cold solution of **7l** (0.54 g, 1 mmol) and *N*-methyl-morpholine (0.11 ml, 1 mmol) in THF (5 ml). The mixture was stirred at 0° for 1 h and another hour at 20°, 1N $NaHCO_3$ (5 ml) was added and the solution concentrated *in vacuo* to about 10 ml. Ethyl acetate (150 ml) was added and work-up continued as before. The product, **7g**, crystallized from ethyl acetate/ether: 0.44 g (64%), m.p. 156°, $[\alpha]_D^{20}$ and TLC. as in preparation a).

$C_{34}H_{49}N_5O_8S$	Calc.	C 59.4	H 7.2	N 10.2	O 18.6	S 4.7%
(687.86)	Found	„ 58.9	„ 7.3	„ 10.0	„ 19.2	„ 5.1%

Boc-Tyr-D-Ala-Gly-MePhe-Met(O)-ol (**7h**). **7g** (0.69 g, 1 mmol) was dissolved in acetic acid (15 ml), cooled to 0°, and 11.2M H_2O_2 (0.1 ml, 1.1 mmol) added. After 1 h at 20°, the mixture was slowly poured into vigorously stirred ether (200 ml) and the product isolated by filtration. It was reprecipitated from chloroform/ether: 0.65 g (92%). $[\alpha]_D^{20} = -25.5°$ ($c = 1.1$, methanol), Rf 0.50 (B), 0.36 (A).

$C_{34}H_{49}N_5O_9S$	Calc.	C 58.0	H 7.0	N 9.9	O 20.5	S 4.6%
(703.9)	Found	„ 57.4	„ 7.7	„ 9.9	„ 19.9	„ 4.5%

Boc-D-Ala-Gly-OH (**7i**). **7c** (33.6 g, 100 mmol) was dissolved in methanol (250 ml), 10% Pd/C (3.5 g) added and the mixture hydrogenated at 3.5 bar in a *Parr* apparatus at 20° for 30 min. The catalyst was removed by filtration through a bed of *Celite*, the filtrate concentrated *in vacuo* and the product **7i** was crystallized by addition of ether and petroleum ether: 21.1 g (86%); m.p. 113° (dec.), $[\alpha]_D^{20} = +9.3°$ ($c = 1$, DMF).

$C_{10}H_{18}N_2O_5$	Calc.	C 48.8	H 7.4	N 11.4	O 32.5%
(246.26)	Found	„ 48.7	„ 7.4	„ 11.2	„ 32.4%

Boc-D-Ala-Gly-MePhe-Met-ol (**7k**). **7i** (7.4 g, 30 mmol) and *N*-ethyl-morpholine (3.3 ml, 30 mmol) were dissolved in THF (70 ml) and cooled to −25°. Isobutyl chloroformate (3.4 ml, 30 mmol) was added followed, after stirring at −20° for 10 min, by a cold solution of H-MePhe-Met-ol, HCl (**7b**) (10 g, 30 mmol) and *N*-methyl-morpholine (3.3 ml, 30 mmol) in DMF (30 ml). After stirring at −15° for 2 h 1M $KHCO_3$ (30 ml) and water (30 ml) were added and the temperature allowed to rise to 0°. After 30 min ethyl acetate (0.4 l) was added and work-up continued as before. The product, **7k**, was precipitated from ethyl acetate by the addition of ether and petroleum ether: 12.9 g (82%); m.p. 53° (dec.), $[\alpha]_D^{20} = -40.3°$ ($c = 0.98$, DMF).

H-D-Ala-Gly-MePhe-Met-ol, TFA (**7l**). **7k** (12.8 g, 24 mmol) was dissolved in methylene chloride (60 ml) and trifluoroacetic acid (60 ml). After 30 min at 20° the mixture was slowly poured into ether (0.6 l) at 0°. The product was isolated by filtration and dried over P_2O_5 and KOH-pellets to give a very hygroscopic form; 10.9 g (83%); m.p. 74° (dec.).

H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol, CH_3COOH (**7**). **7h** (1 g, 1.4 mmol) was dissolved in methylene chloride (3 ml) and trifluoroacetic acid (3 ml) at 0°. After 1 h at 0° the mixture was concentrated *in vacuo*, precipitated with a large volume of ether, isolated by filtration and dried over KOH-pellets. It was dissolved in water (10 ml) and filtered through a column (35 ml) of ion-exchange resin (*Amberlite* IRA 410, in the acetate form). The eluate containing the peptide was lyophilized: 930 mg. $[\alpha]_D^{20} = -6.1°$ ($c = 1$, CH_3COOH 95%). Rf 0.19 (C), 0.33 (D).

Amino acid analysis: Tyr 0.99; Ala 1.05; Gly 0.96; MePhe 1.0 (peptide content, 87% free base).

^{1}H-NMR. (D_2O): 1.2/*d* (Ala-β); 1.7–2.2/*m* (Met(O)-ol-β); 2.7/*s* ($SO-CH_3$); 2.95/*s* ($N-CH_3$); 2.8–3.4/*m* (Tyr-β, MePhe-β, $S-CH_2$); 3.6/*d* (CH_2-OH); 3.9–4.2/*m* (Tyr-α, Gly-α, Met(O)-ol-α); 4.2/*qa.* (Ala-α); 5.1/*m* (MePhe-α); 6.9/*d* (Tyr-arom.); 7.2/*d* (Tyr-arom.); 7.4/*s* (MePhe-arom.). – ^{13}C-NMR. (D_2O): All resonances were assigned, in particular 62.0, 61.8/*d* (MePhe-α); 62.0, 51.6/*d* (Met(O)-ol-α); 33.7, 33.5/*qa* ($N-CH_3$), the split signals being due to the presence of a 1:1 mixture of sulfoxide-isomers.

H-MePhe(p-NO_2)-OH (**25a**). HNO_3, spec. gravity 1.40 (1.4 ml) was added dropwise to a well stirred solution of H-MePhe-OH (3.6 g, 20 mmol) in H_2SO_4, spec. gravity 1.85, (14 ml), while the temperature was kept at 20°. After stirring for 40 min, the mixture was poured onto crushed ice (150 g), the resulting solution was adjusted to pH = 6.6 with conc. ammonia and cooled to −5° for complete precipitation of the product. It was isolated by filtration, washed with water and dried: 3.8 g (85%), yellow; m.p. 155° (dec.), $[\alpha]_D^{20} = +31°$ ($c = 1$, 6N HCl). – UV. (1N HCl): 270 (8750).

Boc-MePhe(p-NO_2)-OH (**25b**). Di(*t*-butyl)pyrocarbonate (*Fluka,* 9.4 ml, 43 mmol) was added to a solution of **25a** (3.2 g, 14 mmol) in 1N $KHCO_3$ (12 ml) and *t*-butyl alcohol (26 ml). The pH of the mixture was kept at pH = 8–9 by addition of Na_2CO_3. After 2 days at 20°, water (80 ml) was added and the mixture extracted with ether (50 ml). The aqueous phase was adjusted to pH = 2 by the addition of 1N HCl at 0° and extracted twice with methylene chloride (100 ml). The organic layer was washed with 30% NaCl-solution, dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo:* 3.2 g (71%), $[\alpha]_D^{20} = -73.9°$ ($c = 1.1$, CH_3COOH 95%).

$C_{15}H_{20}N_2O_6$	Calc.	C 55.5	H 6.2	N 8.6	O 29.6%
(324.33)	Found	„ 54.9	„ 6.2	„ 8.7	„ 30.0%

H-Tyr-D-Ala-Gly-MePhe-Met-ol, TFA (**26**). **7g** (0.28 g, 0.41 mmol), thioanisole (0.5 ml, 4.2 mmol) and 2-mercaptoethanol (0.1 ml, 1.4 mmol) were dissolved in methylene chloride (1 ml), cooled to 0°, and trifluoroacetic acid (10 ml) added. After 1 h at 20° the mixture was concentrated *in vacuo,* then slowly poured into ether (100 ml) and the precipitate collected by filtration. It was reprecipitated from methanol/methylene chloride/ether, redissolved in water and lyophilized: 180 mg. $[\alpha]_D^{20} = -4.2°$ ($c = 0.25$, CH_3COOH 95%). Rf 0.40 (C), 0.58 (D).

Amino acid analysis: Tyr 1.01; Ala 0.98; Gly 1.02; MePhe 0.9 (peptide content, 88% free base).

^{1}H-NMR. (D_2O): 2.1/*s* ($S-CH_3$); all other resonances as for **7**.

Boc-Tyr-D-Ala-Gly-MePhe-Met(O_2)-ol (**27a**). **7g** (0.26 g, 0.37 mmol) was dissolved in acetic acid (3 ml) and 11.2M H_2O_2 (0.4 ml, 4.5 mmol) added. After 24 h at 20° ether was added to the mixture and the precipitate isolated by filtration. The crude product was purified by column chromatography on silica gel 0.06–0.2 mm *Merck* and eluted with methylene chloride/methanol/acetic acid 85:15:1. Fractions were checked by TLC., pooled and solvents removed *in vacuo:* 210 mg. $[\alpha]_D^{20} = -34°$ ($c = 0.93$, DMF). Rf 0.69 (B), 0.51 (A).

H-Tyr-D-Ala-Gly-MePhe-Met(O_2)-ol, TFA (**27**). **27a** (0.18 g, 0.25 mmol) and anisole (0.3 ml, 2.8 mmol) were dissolved in methylene chloride (2 ml), cooled to 0° and trifluoroacetic acid (10 ml) added. After 30 min at 20° the mixture was concentrated *in vacuo*, then slowly poured into ether (100 ml) and the precipitate isolated by filtration. It was reprecipitated from methanol/ether, redissolved in water and lyophilized: 174 mg $[\alpha]_D^{20} = -3.4°$ ($c = 1.7$, CH_3COOH 95%). Rf 0.28 (C), 0.39 (D).

Amino acid analysis: Tyr 1.01; Ala 1.00; Gly 0.99; MePhe 0.9 (peptide content, 74% free base).

^{1}H-NMR. (D_2O): 3.1/*s* (SO_2-CH_3): all other resonances as for **7**.

S-*Methyl-Cys-ol* (**29a**). *S*-Methyl-Cys-OMe, HCl [52] (3.7 g, 20 mmol) dissolved in water (25 ml)/ ethanol (25 ml) was added dropwise to a well stirred solution of $NaBH_4$ in water (25 ml)/ethanol (25 ml) at 0°. After 4 h at 0° and 18 h at 20° the mixture was filtered, concentrated *in vacuo* to a small volume and extracted twice with chloroform (200 ml). The chloroform layer was washed with a small volume of 30% NaCl-solution, dried with anhydrous Na_2SO_4 and evaporated *in vacuo:* 1.8 g (74%), colourless, viscous liquid. An oxalate crystallized from ethanol: m.p. 146° (dec.), $[\alpha]_D^{20} = -30.8°$ ($c = 1.1$, water).

$C_6H_{13}NO_5S$	Calc.	C 34.1	H 6.2	N 6.6	O 37.9	S 15.2%
(229.25)	Found	„ 34.1	„ 5.9	„ 6.7	„ 37.7	„ 15.6%

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