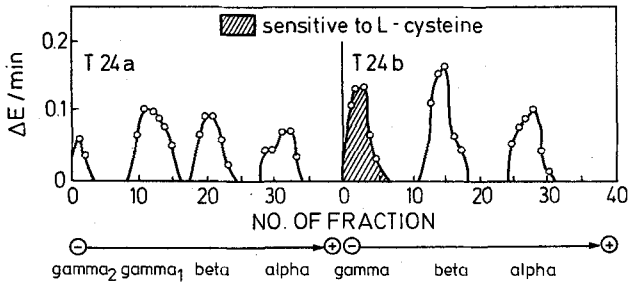

Figure 2. A comparison of pyruvate kinase isoenzymes from chromatin extracts of T24 two sublines:the non-tumorigenic T 24a and the tumorigenic T 24b. Hatched area: isoenzyme sensitive to L-cysteine.

metabolism[1,2]. The sensitivity to L-cysteine also points to changes in the primary structure of PK, and thus in the cell genotype, which take place during a multistage process of carcinogenesis.

The altered sensitivity of tumor PK to normal signal molecules, such as ATP[3,22] or fatty acids[23], also brings about several metabolic consequences[24].

It can be concluded that the decrease in electrophoretic mobility of the slow-migrating PK gamma isoenzyme and the appearance of its sensitivity to L-cysteine could be used as cellular markers of immortalization and tumorigenic transformation, respectively, in human urothelial cell lines. It seems that the two coupled features, tumorigenicity in nude mice and sensitivity to L-cysteine, might be useful in monitoring successive stages of carcinogenesis in vitro.

Since numerous tumor markers present in the body fluids can be used for laboratory diagnostics of neoplastic diseases in living organisms[25], a new attempt has been made to evaluate the described PK isoenzyme sensitive to L-cysteine for this purpose as well, using in vivo studies.

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Synthesis of $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2, Hyp^5] enkephalin amide, a new highly potent analgesic enkephalin-related glycosyl peptide

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Summary. A new series of O-glycosyl enkephalins has been prepared, following a convergent strategy, with high chemical yields. The galactosyl analogue, $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2, Hyp^5] enkephalin amide proved to be one of the most potent in vivo opioid agonists synthesized up to now.

Key words. Analgesic peptides; enkephalin analogues; galactosylpeptides; glycosylpeptides; peptide synthesis.

The search for more potent and selective biologically active analogues is one of the main goals in peptide chemistry. This aim has been pursued in our laboratory by producing different modifications of the enkephalins[1,2], two pentapeptides originally discovered by Hughes et al.[3]. The promising results obtained by

incorporation of sugar moieties into the fifth position of an enkephalin analogue[4,5], and the increasing evidence of the important role played by glycopeptides and the carbohydrate-containing fragments of glycoproteins[6], led us to further work in this direction. As a result, we were able to synthesize a very potent glycosylated enkephalin analogue.

For the O-glycosylation of peptides, a stepwise strategy starting at the C-terminus and proceeding towards the N-terminus has normally been recommended[7]. In view of the fact that the N-terminal fragment 1–4 and the amino acid 5 have a different influence on the activity and selectivity of enkephalins[8,9], a convergent strategy was preferred in order to modify the two significant parts of the peptide separately.

One of the series of glycosylated enkephalins thus obtained involves the synthesis of the basic tetrapeptide Tyr-DMet-Gly-Phe and various glycosylated amino acid moieties. The parent compound of this series is [DMet^2, Pro^5] enkephalin amide (**6**) which has been described as being a potent μ-agonist[10]. The substitution of Pro^5 by Hyp^5 and the O-glycosylation of this position by both glucose and galactose have produced different in vivo active opioid agonists, namely [DMet^2, Hyp^5] enkephalin amide (**7**), $O^{1,5}$-(β-D-glucopyranosyl) [DMet^2, Hyp^5] enkephalin amide (**8**) and $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2, Hyp^5] enkephalin amide (**9**)[11]. The O-galactosyl derivative (**9**) has proved to be 50,000-fold more potent than morphine in male Sprague-Dawley rats in the Tail Immersion and Paw Pressure tests of analgesia, after intracerebroventricular administration. On the other hand, the glucosyl analogue was only 30-fold more potent than morphine in the same test[12].

Synthesis

The reaction pathways corresponding to the synthesis of the non-glycosylated peptides and the glycosyl analogues are shown in scheme 1 and scheme 2, respectively. Chemical yields and chromatographic data corresponding to the protected intermediates and the deprotected final analogues are summarized in the table.

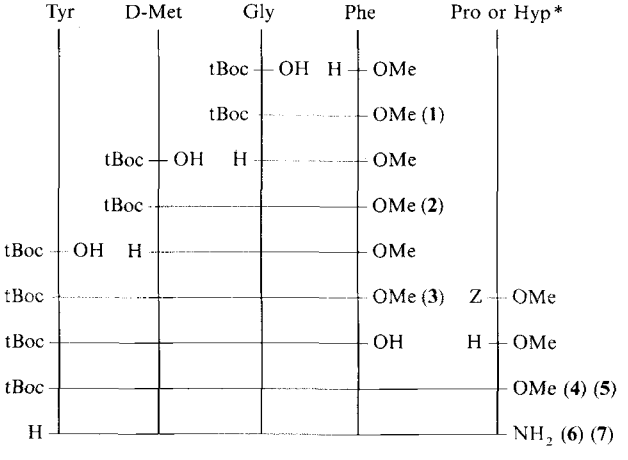
The tetrapeptide tBoc-Tyr-DMet-Gly-Phe-OMe was obtained by the stepwise procedure starting from H-Phe-OMe and using the mixed anhydride/isobutylchloroformate equimolar procedure. The parent compound [DMet^2, Pro^5] enkephalin amide and the Hyp^5-analogue were obtained by incorporation of the C-protected fifth amino acid using the DCC/HOBt procedure. The N-terminal tBoc protecting group was cleaved in TFA/CH_2Cl_2 (1:1) and the C-terminal amide was formed from the methyl ester by treatment with NH_3/MeOH (saturated at 0 °C).

The βO-glycosylation of Z-Hyp-OMe was performed by the Koenigs-Knorr procedure[13] using 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide and $Hg(CN)_2$ as acid acceptor in C_6H_6/nitromethane at 80 °C (40–50% chemical yield). The sugar was added in three portions at different times in order to avoid its decomposition under the conditions used. The trifluoromethanesulphonic anhydride procedure (– 70 °C, benzylated sugars)[14] was also followed (40% chemical yield β anomer).

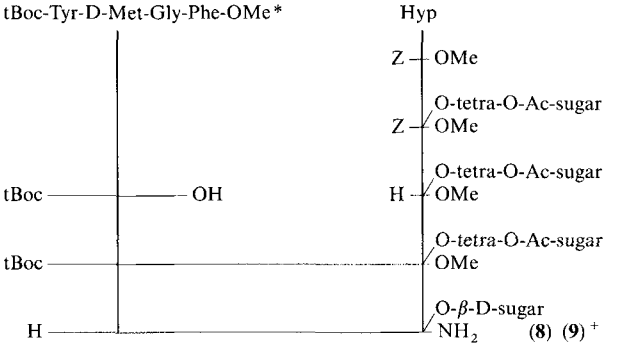
Chemical yields and chromatographic data of the protected intermediates and the final enkephalin analogues. T.l.c. solvent systems are (a) HCCl$_3$/MeOH/AcOH (95:5:3) and (b) AcOEt (compounds **1–4**); Butanol/AcOH/H_2O (4:1:1) (compounds **5, 8, 9**); AcOEt/pyridine/AcOH/H_2O (60:20:6:11) (compounds **6, 7**). H.P.L.C. values are given for the following conditions: 10 μm C_{18} 250 × 4.6 mm column elution H_2O-0.05% TFA/CH_3CN; Compounds (**1–5**): Isocratic mode elution (70:30) flow rate 1 ml/min; Compounds (**6–9**): Gradient mode elution 10 to 100% CH_3CN during 30 min, flow rate 0.9 ml/min. λ = 254 nm.

Compound*	Yield (%)	T.l.c. Rf_a	Rf_b	HPLC R.T.	k
(**1**)	98	0.3	0.6		
(**2**)	98	0.4	0.5	16.6	7.3
(**3**)	70	0.35	0.35	15.2	6.6
(**4**)	74	0.35	0.4	14.4	6.2
(**5**)	55	0.1	0.3	10.0	4.0
(**6**)	91		0.5	15.9	5.1
(**7**)	90		0.4	14.1	4.4
(**8**) & (**9**)	90		0.3	13.0	4.0

* Compounds corresponding to each number are indicated in the text as well as in schemes 1 and 2. Purification of the protected intermediates was carried out by flash chromatography, using Silica gel (40–63 μm), 15 × 2 cm or 15 × 5 cm columns eluted with AcOEt at a flow rate of 5 cm/min.



Scheme 1. * (**4**) (**6**) Prolyl peptides; (**5**) (**7**) Hydroxyprolyl peptides.



Scheme 2. * Tetrapeptide synthesized as indicated in scheme 1; + (**8**) Glucopyranosyl derivative; (**9**) Galactopyranosyl derivative.

The yield obtained in the incorporation of the protected glycosyl hydroxyproline derivatives into the rest of the enkephalin-related sequence by the DCC/HOBt procedure depended on the hydroxyl protecting group used. The best yields were obtained by using acetylated sugars instead of the bulkier benzyl derivatives. Thus in the coupling between O^1-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl) hydroxyproline methyl ester and tBoc-Tyr-DMet-Gly-Phe-OH, a 75% yield was obtained compared to 29% using benzylated galactose methyl ester.

The N-terminal tBoc protecting group was cleaved in TFA/CH_2Cl_2 (1:1). The cleavage of acetyl groups and the terminal amide formation from the methyl ester were carried out at the same time by treatment with NH_3/MeOH (saturated at 0 °C). The resulting glycopyranosyl peptides were then purified by gel filtration on Sephadex G-25 and semipreparative HPLC, C_{18}, solvent system H_2O-0.05% TFA/CH_3CN, isocratic mode elution 70:30, $\lambda = 280$ nm. Homogeneity was assessed by t.l.c. and a reversed phase HPLC ODS 5 µm column eluted with 0.05% aqueous TFA/acetonitrile by a gradient from 9 to 100% acetonitrile at a linear rate of 3.0% ACN/min with absorbance – ratio measurements. Characterization was ascertained by amino acid analysis; optical rotatory dispersion (**8**) $[\alpha]^{20}_{295}$ 2.33°, $[\alpha]^{20}_{254}$ 11.66°, $[\alpha]^{20}_{240}$ 27.0°, $[\alpha]^{20}_{235}$ 20.0°, (**9**) $[\alpha]^{20}_{295}$ 4.37°, $[\alpha]^{20}_{254}$ 0°, $[\alpha]^{20}_{240}$ 19.37°, $[\alpha]^{20}_{235}$ 9.69° (c = 0.32, MeOH); ^{1}H-n.m.r. (270 MHz, DMSO-d_6) (**8**) δ = 3.83 CαH Tyr, 2.82 & 2.72 CβH Tyr, 6.96 & 6.65 o & m arom Tyr, 4.28 CαH D-Met, 1.76 & 1.61 CβH D-Met, 2.18 Cγ H D-Met, 1.96 Cδ H D-Met, 3.70 & 3.52 CαH Gly, 4.66 CαH Phe, 3.00 & 2.68 CβH Phe, 4.27 CαH Hyp, 2.17 & 1.95 CβH Hyp, 4.40 Cγ H Hyp, 3.82 & 3.69 CδH Hyp, 4.35 H1 glcp ($J_{1,2}$ = 7.6), 3.20–3.26 H2, H3, H4, H5 glcp, 3.59 H6 glcp; (**9**) δ = 4.14 H1 galac ($J_{1,2}$ = 7.1), 3.20–3.26 H2, H3, H4, H5 galac, 3.59 H6 galac, and FAB-mass spectrometry m/e positive ions 791 M + H, 813 M + 23, 483, 353.

Racemization was checked by GC on an enantioselective stationary phase (cyano ethyl-siloxane-L-valine-S-α-phenyl ethyl amide, Quirasil-valine). The extent of racemization produced during the steps outlined in scheme 1 both for DCC/HOBt and mixed anhydride methods was not greater than 1%.

In summary, the above-described synthesis of O-glycosyl enkephalin derivatives has enabled us to obtain, with high chemical yields, a new series of potent antinociceptive glycosyl peptides. The galactosyl analogue exhibited by far the greatest activity, being one of the most potent in vivo opioid peptide agonists synthesized up to now. Moreover, significant differences in activity have been found depending on the type of sugar moiety present in the molecule, which demonstrates the importance of fine structure for the biological action of such derivatives. In the future, the introduction of sugar moieties into the position 5 of enkephalins could be a potent tool to improve their analgesic activity. An easy and versatile strategy for the synthesis of such analogues, producing high yields, was established in this work.

Footnote. The following abbreviations according to the IUPAC-IUB Commission have been used: HOBt, 1-hydroxybenzotriazole; DCC, N,N-dicyclohexylcarbodiimide; MA, mixed anhydride procedure; tBoc, tert.-butyloxycarbonyl; Z, benzyloxycarbonyl.

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