

Synthesis of 3-*O*-Carboxyalkyl Morphine Derivatives and Characterization of Their Acid-Base Properties

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The C-3 phenolic hydroxy group containing morphine derivatives (morphine, oxycodone, naloxone, naltrexone) are excellent candidates for the synthesis of 3-*O*-functionalized molecules. Achieving free carboxylic group containing derivatives gives the opportunity for further modification and conjugation that could be used for immunization and immunoassays. For this purpose ethyl bromo- and chloroacetate can be used as *O*-alkylating agents. Hydrolyzing the products affords the appropriate free carboxylic group containing 3-*O*-carboxyalkyl derivatives. As these molecules contain an acidic and a basic functional group the protonation macro- and microconstants were determined too, using pH-potentiometry and NMR-pH titration, beside fully characterizing their structure using IR, CD, NMR and HR-MS measurements.

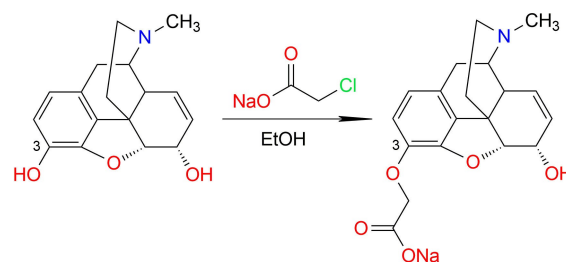
Keywords: morphine, O ligands, haptens, protonation, NMR spectroscopy.

Introduction

The C-3 phenolic hydroxy group in morphine derivatives is an important structural feature for analgesic activity. *O*-Alkylation of this hydroxy group results in compounds (codeine, ethylmorphine) which are less potent than morphine. However, codeine is used as analgesic to relieve mild to moderate pain besides possessing antitussive activity.

Numerous *O*-alkylated derivatives of morphine have been prepared and the C-3 carboxymethyl ether proved to be very important in the development of radioimmunoassay of morphine. One of the very first 3-*O*-derivatives of morphine was reported by Spector and Parker which was a 3-*O*-alkylated compound.^[1,2] Morphine base was treated with sodium chloroacetate in ethanol to obtain 3-*O*-carboxymethylmorphine (2-(morphin-3-*O*-yl)acetic acid) (Scheme 1). The product did not have any analgesic effect but coupling with bovine serum albumin (BSA) was possible in the

presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide. The conjugate was immunogenic and it was used for the quantitative determination of morphine. Rabbits were immunized and blood was collected after two months. To follow the binding and inhibition of morphine to the antigens [³H]-dihydromorphine was used. Both the radioactive and nonradioactive molecules show competitive binding to the antibodies present in the antisera. Using known amount of labeled and unlabeled morphine a standard curve can be obtained and determination of unknown quantities of morphine is possible. This method allowed the determination of morphine in



Scheme 1. Synthesis of 2-(morphin-3-*O*-yl)acetic acid.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbdv.202100135>

biological fluids without the necessity of extraction procedures. The assay is very sensitive for morphine, in serum 50 to 100 picograms of morphine can be determined in 0.5 mL assay mixture. Rubinstein and Ullman prepared the same conjugate but used the mixed anhydride method with isobutyl chloroformate.^[3]

Buechler boiled morphine with bromoacetic acid in ethanol to obtain 2-(morphin-3-O-yl)acetic acid.^[4] Heimann et al. treated the ethanol solution of potassium morphinate with ethyl bromoacetate. The received ethyl 2-(morphin-3-O-yl)acetate was hydrogenated in the presence of Pd/C catalyst and after hydrolysis 2-(dihydromorphin-3-O-yl)acetic acid was obtained.^[5]

Gandhi et al. treated the acetonitrile solution of 6-monoacetylmorphine with chloroacetic acid in the presence of sodium hydroxide.^[6] 6-O-Acetyl ester of 2-(morphin-3-O-yl)acetic acid was coupled to bovine serum albumin and this immunogen generated antibodies against heroin and its metabolites. The antibody was labelled with fluorescein isothiocyanate in order to develop a fluorescence immunoassay. Heroin and its metabolites can be measured in biofluids up to 0.01 ng/mL concentrations.

Ethyl 2-(morphin-3-O-yl)acetate was synthesized by Heiman, Raden and Dubler.^[5] In their patent morphine was dissolved in absolute ethanol and treated with potassium ethoxide. Ethyl bromoacetate was added and after the reaction the product was purified using preparative thin layer chromatography. This compound later was used by Spiehler,^[7] Spector,^[8] Findlay^[9] and Hand^[10] for quantitative measurement of morphine and its metabolites, using various immunoassay methods.

All of the so far mentioned derivatives possess the ability to conjugate them to a bigger carrier molecule and induce immune reaction. Hence these are haptens by definition. Drug molecules solely do not induce the defense of the immune system. Haptens are still not immunogenic, however, these compounds have the structural moiety (carboxylic/amino group) to conjugate them to antigenic carrier molecules such as proteins, lipids, or polysaccharides. This gave the idea of vaccination against drug abuse that could be an alternative therapy beside the methadone protocol.^[11,12] The successful application of these conjugate vaccines mainly relies on hapten design and density, coupling strategy and selection of carrier protein along with vaccine adjuvant.^[13,14]

A new trend can be observed in the case of functionalization of the aromatic ring. Replacing the

phenolic hydroxy group with an isosteric amino group leads to amides. These derivatives are more resistant against hydrolysis than the ester equivalents. The synthesis and conjugation of these amide haptens to tetanus toxoid was reported by Li et al.^[15] PrOxyHap is a C3 amino group containing hapten acylated with β -mercaptopropionic acid and the C6 position is modified into a 2-oxopropyl moiety (Figure 1). After tetanus-toxoid conjugation mice were immunized. The antinociceptive effects of heroin, morphine and 6-O-acetylmorphine were inhibited in the animals by antigens.

Other heroin-like haptens were designed by Matyas et al.^[16] One of these compounds is the 3-amino group containing 6-O-acetylmorphine derivative called 6-AcMorhap. The amino group is acylated with β -mercaptopropionic acid, just like in PrOxyHap. 6-AcMorHap produced high titers of antibodies in mice along with other derivatives.

During the metabolism of morphine and its derivatives functionalization of the 3-OH group could happen while 3-O-glucuronides are formed. All of these metabolites are amphoteric as they contain originally a tertiary amino group and as a result of glucuronide conjugation a free carboxylic group, too. Although these metabolites are not as active as the parent molecules, they still possess some pharmacological effects. Moreover, they can be used for immunoanalytical purposes to determine the amount of morphine in urine.^[17,18]

Our research team has recently published the synthesis and characterization of acid-base properties of *N*-carboxyalkyl and *N*-acetylglycine morphine derivatives.^[19] The purpose of the recent work was to extend our studies for the synthesis of 3-O-carboxymethyl derivatives. These functionalized compounds contain basic tertiary nitrogen and free carboxylic groups, so we intended to determine their ionization constants, too. The synthesized molecules are not only potential candidates for different type of immunoassays but also show similarity to morphine-

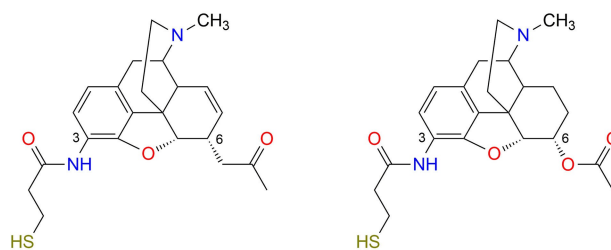


Figure 1. PrOxyHap and 6-AcMorHap.

3-glucuronide as amino and carboxy group containing zwitterionic compounds.

Results and Discussion

Hapten Synthesis

The synthesis of 3-O-functionalized morphine derivative haptens was designed. For this purpose morphine (**1**), oxymorphone (**2**), naloxone (**3**) and naltrexone (**4**) were selected as starting materials.

Metallic sodium was dissolved in absolute ethanol and morphine hydrochloride salt was added to obtain the sodium salt of morphine. Then it was mixed with ethyl bromoacetate and refluxed for 6 h. The conversion of the reaction was monitored by thin layer chromatography (TLC). After the work-up process, ethyl 2-(morphin-3-O-yl)acetate (**5**) was isolated (Scheme 2).

Treatment of oxymorphone with ethyl chloroacetate in acetone solution for 12 h reflux in the presence of potassium carbonate and potassium iodide yielded ethyl 2-(oxymorphon-3-O-yl)acetate (**6**) (Scheme 3).

The preparation method of naloxone derivative was identical with that of oxymorphone. After dissolving the base in acetone ethyl chloroacetate was added in the presence of potassium carbonate

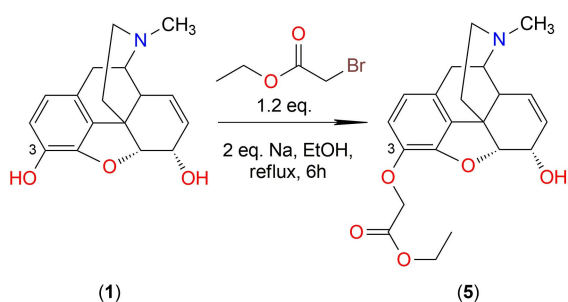
and potassium iodide and the mixture was refluxed for 12 h (Scheme 4). After work-up, ethyl 2-(naloxon-3-O-yl)acetate (**7**) was obtained.

The same reaction was accomplished with naltrexone (Scheme 5). The product was ethyl 2-(naltrexon-3-O-yl)acetate (**8**).

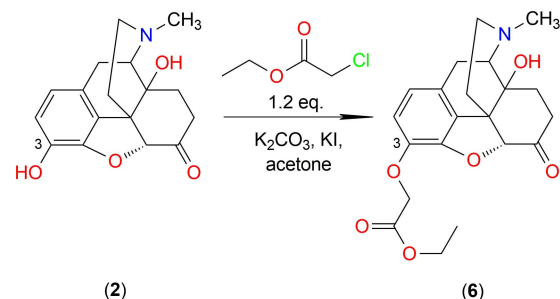
All of the esters were hydrolyzed in the same way. The compounds were dissolved in a minimal amount of ethanol, the solutions were stirred and heated with aqueous sodium hydroxide. After conversion was complete the pH was adjusted to 2–3 and the products were obtained as hydrochloride salts (Scheme 6).

Structures of the products were confirmed with 1D and 2D NMR measurements along with HR-MS analysis. The chemical shifts of the new side chains are summarized in Table 1.

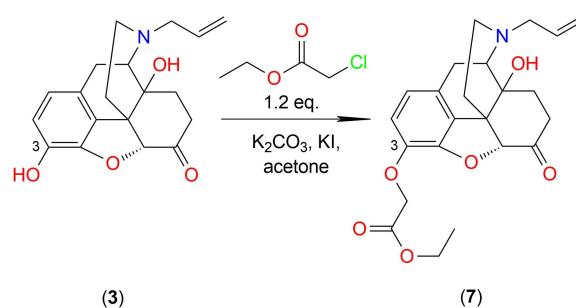
Circular dichroism (CD) spectroscopy is widely used in the analysis of proteins and small molecule pharmaceuticals. Several opium alkaloids were characterized by Purdie et al. using CD.^[20] Characteristic structural bands can be easily observed on the morphine and naloxone spectra. Approximately at 300 nm the negative band of n- π^* transition of the C-6 oxo derivative can be detected. The allyl group of naloxone and cyclopropylmethyl group of naltrexone do not have significant effect on the spectra. Around at 240 nm and 275 nm a positive band-pair can be



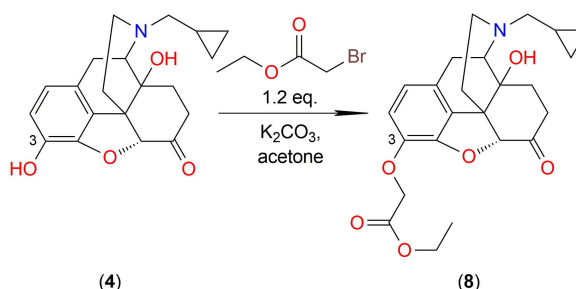
Scheme 2. Synthesis of ethyl 2-(morphin-3-O-yl)acetate.



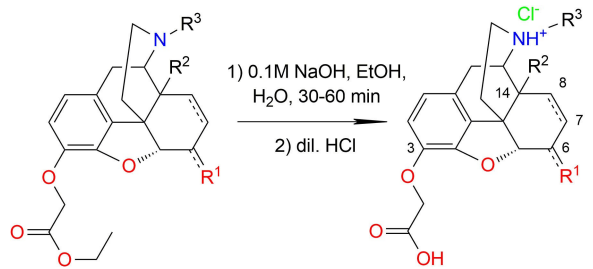
Scheme 3. Synthesis of ethyl 2-(oxymorphon-3-O-yl)acetate.



Scheme 4. Synthesis of ethyl 2-(naloxon-3-O-yl)acetate.



Scheme 5. Synthesis of ethyl 2-(naltrexon-3-O-yl)acetate.



Substrate	Product	R ¹	R ²	R ³	Δ _{7,8}
(5)	(9)	-OH	-H	-CH ₃	=
(6)	(10)	=O	-OH	-CH ₃	-
(7)	(11)	=O	-OH	allyl	-
(8)	(12)	=O	-OH	cyclopropylmethyl	-

Scheme 6. Hydrolysis of 3-*O*-hapten esters.

observed. The intensity and ratio of these are different in the synthesized derivatives: in the esters the peak is higher at 270 nm, while in the acids it is higher at 240 nm.

In the case of the C-6 hydroxy group containing morphine derivatives a weak band appears in the ¹L_b range of π-π* transition that can be assigned to the substituted aromatic ring around 285 nm. Based on the theoretical background of the sign of L_b-band described by Snatzke, alkaloids with morphine skeleton have negative sign.^[21] Furthermore an ¹L_a π-π* transition can be assigned to the substituted aromatic system, too. The presence of these bands and comparing them to the standard spectra give unambiguous evidence to the structural analogy.

At the range of 210–218 nm a characteristic *p*-band for the aromatic ring is present in every derivative.

Protonation Constants

The pharmacokinetic behavior of biomolecules and drugs are influenced by several physicochemical properties, the most important ones are the acid-base character, lipophilicity, permeability, and solubility.^[22] The ionization state of a molecule in solution is determined by its acid-base character and the pH of the solution. In turn, the ionization state influences all pharmacokinetic properties.^[23]

3-*O*-carboxymethyl-opioid compounds with a free carboxy site have two basic functional groups, namely a carboxylate and an amino group, and can occur in four different microscopic protonation forms in solution. Such compounds are anionic in alkaline solutions and upon protonation are converted into either a zwitterionic or an uncharged form. These two forms, differing from each other only in the site of protonation, are called protonation isomers. The second protonation step produces a cation. These four microscopic protonation forms and the respective protonation microconstants are shown in Scheme 7 on the example of 2-(morphin-3-*O*-yl)acetic acid.

Protonation Macroconstants of 3-*O*-haptens

We have recently investigated *N*-carboxymethyl opioid compounds,^[19] where we showed that the basicity of the carboxylate site is smaller by several orders of magnitude than that of the amino site, thus *K*₂ values of the dibasic 3-*O*-carboxymethyl compounds characterize almost exclusively the protonation of the carboxylate sites, with *K*₂ practically equal to *k*_N^C.

The determination of protonation constants is usually carried out by pH-potentiometry.^[24] We also used this method to obtain the protonation constants of 3-*O*-haptens. However, log *K*₂ values proved to be too small, so we carried out NMR-pH titrations, where the exact pH in highly acidic solutions was shown by indicator molecules.

Table 1. ¹H- and ¹³C-NMR assignment of C3-substitutes of 3-*O*-carboxymethyl morphine derivatives.

	(D ₆)DMSO		D ₂ O		
	CH ₂ O–C(3) (¹ H/ ¹³ C) ppm	C=O (¹³ C) ppm	CH ₂ O–C(3) (¹ H/ ¹³ C) ppm	C=O (¹³ C) ppm	
Ethyl 2-(morphin-3- <i>O</i> -yl)acetate (5)	4.72/66.2	169.5	2-(Morphin-3- <i>O</i> -yl)acetic acid HCl (9)	4.75/68.9	176.5
Ethyl 2-(oxymorphon-3- <i>O</i> -yl)acetate (6)	4.82/65.9	169.2	2-(Oxymorphon-3- <i>O</i> -yl)acetic acid HCl (10)	4.81/69.4	177.0
Ethyl 2-(naloxon-3- <i>O</i> -yl)acetate (7)	4.81/65.8	168.6	2-(Naloxon-3- <i>O</i> -yl)acetic acid HCl (11)	4.82/69.5	176.8
Ethyl 2-(naltrexon-3- <i>O</i> -yl)acetate (8)	4.77/65.7	168.7	2-(Naltrexon-3- <i>O</i> -yl)acetic acid HCl (12)	4.79/69.5	177.2

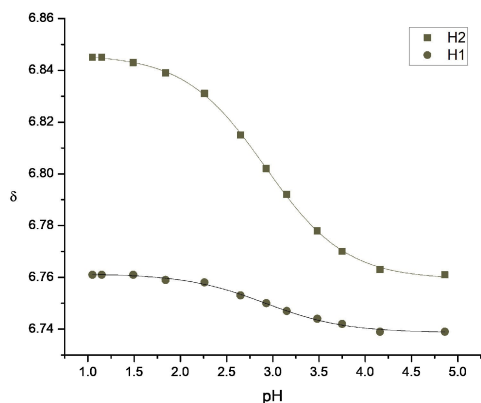
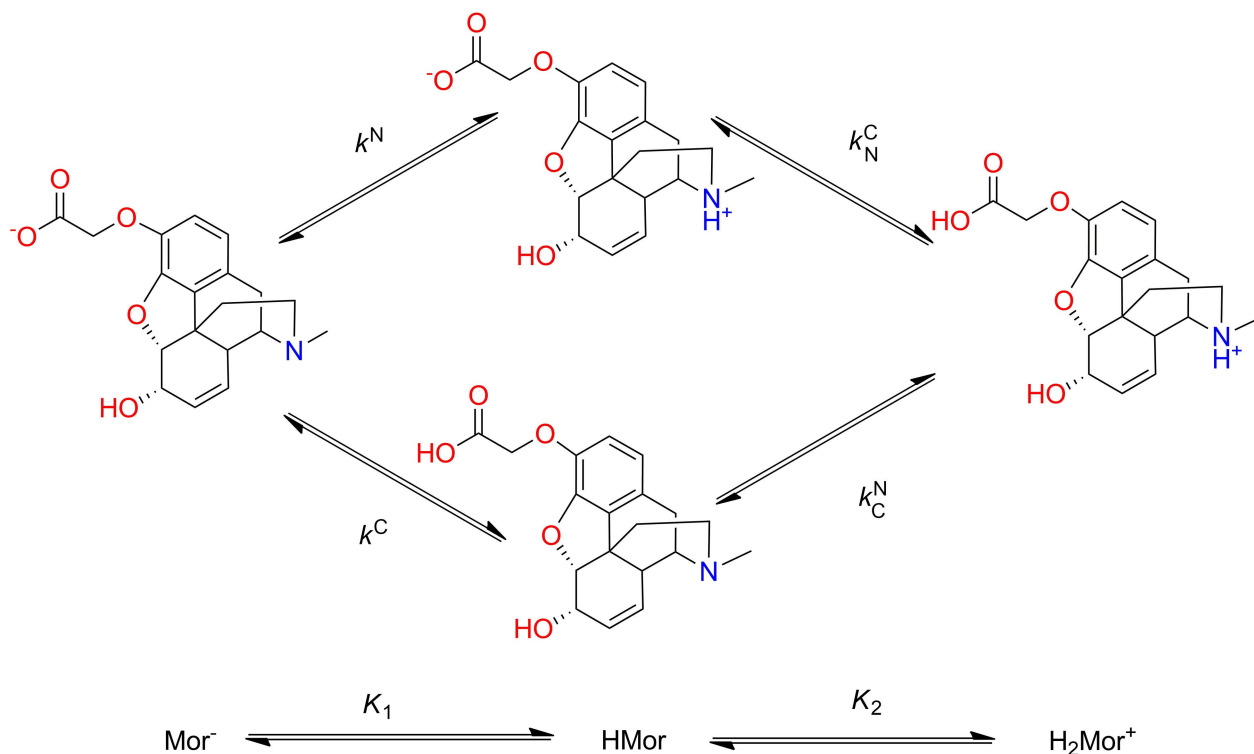


Figure 2. NMR-pH titration curves of the H² and H¹ aromatic protons of 2-(morphin-3-O-yl)acetic acid. Computer fits for log K_2 are shown in solid lines.

Although these compounds possess around twenty protons, the chemical shift of only those protons changes significantly in the acidic pH region that are in the vicinity of the carboxy group. We typically followed the doublets of the aromatic H¹ and H² protons and the signals of the methylene protons in the carboxymethyl side chain.

The NMR-pH titration curve of 2-(morphin-3-O-yl)acetic acid can be seen in *Figure 2* and *Figure 3*.

The protonation macroconstants are shown in *Table 2*.



Scheme 7. The microscopic protonation forms and microspecies of 2-(morphin-3-O-yl)acetic acid.

Table 2. Protonation macroconstants of the investigated 3-O-haptens. The standard deviations are in parentheses.

	$\log K_1$	$\log K_2$
2-(Morphin-3-O-yl)acetic acid (9)	8.32 (0.03)	2.94 (0.02)
Ethyl 2-(morphin-3-O-yl)acetate (5)	8.22 (0.04)	
2-(Oxymorphon-3-O-yl)acetic acid (10)	9.00 (0.04)	2.86 (0.03)
Ethyl 2-(oxymorphon-3-O-yl)acetate (6)	8.87 (0.03)	
2-(Naloxon-3-O-yl)acetic acid (11)	8.35 (0.03)	2.93 (0.03)
Ethyl 2-(naloxon-3-O-yl)acetate (7)	8.23 (0.04)	
2-(Naltrexon-3-O-yl)acetic acid (12)	9.28 (0.04)	3.00 (0.04)
Ethyl 2-(naltrexon-3-O-yl)acetate (8)	9.13 (0.03)	

Table 3. Protonation microconstants and pair-interactivity parameters of the dibasic 3-O-haptens.

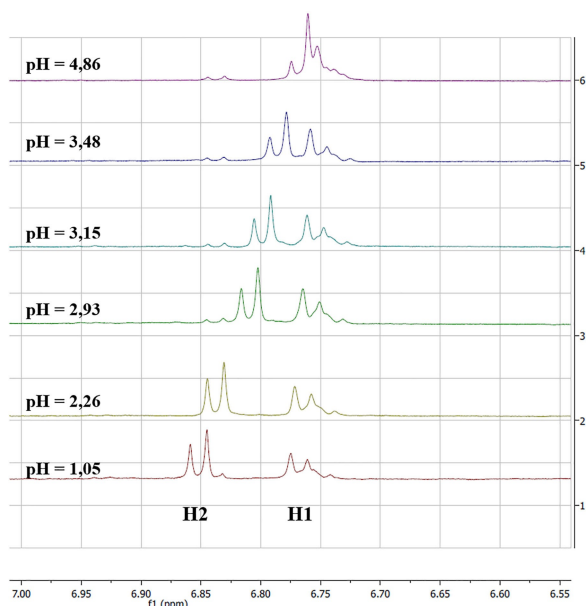
	$\log k^N$	$\log k^C$	$\log k_C^N$	$\log k_N^C$	$\log E_{C,N}$
2-(Morphin-3-O-yl)acetic acid (9)	8.32	3.04	8.22	2.94	0.10
2-(Oxymorphon-3-O-yl)acetic acid (10)	9.00	2.99	8.87	2.86	0.13
2-(Naloxon-3-O-yl)acetic acid (11)	8.35	3.05	8.23	2.93	0.12
2-(Naltrexon-3-O-yl)acetic acid (12)	9.28	3.15	9.13	3.00	0.15

Protonation Microconstants of 3-O-haptens

The dibasic compounds possess a carboxylate and a much more basic amino group; thus the deductive method is needed for the determination of protonation microconstants. This method makes use of a model compound to mimic a microspecies on the minor protonation pathway.^[23] The k_C^N microconstants of the dibasic 3-O-haptens can be mimicked by the macroconstants of their esters. Using this microconstant and the already known two macroconstants, the remaining three microconstants can be calculated for the dibasic 3-O-haptens (Table 3). Table 3 also contains the $\log E_{C,N}$ pair-interactivity parameter that shows to what degree the protonation of a basic group decreases the basicity of the other group.

$$\log E_{C,N} = \log k^N - \log k_C^N = \log k^C - \log k_N^C \quad (1)$$

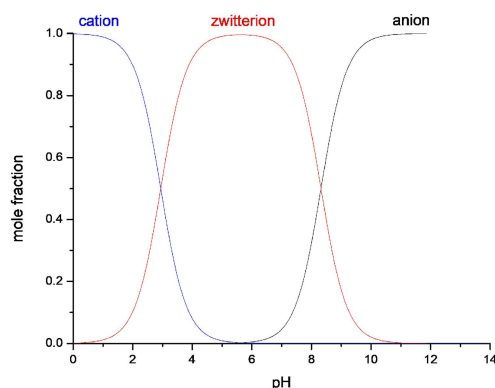
As the basicity of the amino group is larger by several orders of magnitude than that of the carbox-

**Figure 3.** Chemical shifts of the H² and H¹ aromatic protons of 2-(morphin-3-O-yl)acetic acid during NMR-pH titration.

ylate group, the upper protonation pathway of Scheme 7 is the dominant one, and the concentration of the zwitterionic species is much larger than that of the uncharged one. The concentration of the microspecies of the dominant protonation pathway can be shown in distribution diagrams, exemplified here for 2-(morphin-3-O-yl)acetic acid (Figure 4).

The intersections of the species distribution curves indicate that below pH 2.94 the cationic, above pH 8.32 the anionic form is dominant, whereas in-between this compound can mainly be found in the zwitterionic form.

The basicity of the carboxylate site is similar in each molecule, the largest difference is 0.16 in log units. The similar basicity can be explained by the fact that this group is located far away from the opioid skeleton, where the investigated compounds differ from each other. On the other hand, the basicity of the amino site shows larger variations, the largest difference is 0.96 in log units. The differences in the basicity of the amino groups can be explained by inductive and field effects from moieties situated in the vicinity. The replacement of the *N*-methyl group with an *N*-allyl one lowers the basicity of the amino group in the oxymorphone-naloxone pair, but the cyclopropylmethyl substituent causes the opposite effect in the oxymorphone-naltrexone pair. The

**Figure 4.** The microspecies distribution diagram of 2-(morphin-3-O-yl)acetic acid.

comparison of the oxymorphone-morphine pair shows that the hydroxy group at C-14 increases the basicity of the nitrogen atom, despite the presence of the electron withdrawing keto group. Intramolecular hydrogen bonding between the tertiary amino and hydroxy groups partly accounts for the increased basicity of the amino group. Saturation of the C7–C8 double bond also contributes to the higher amino basicity of oxymorphone, as it increases the electron density around the nitrogen atom.

The carboxylic acids show a higher amino basicity than their ester derivatives because the former are predominantly in their negatively charged carboxylate form at the pH of amino protonation, whereas the uncharged ester groups exert a strong electron withdrawing effect. The $\log E_{C,N}$ pair-interactivity parameters are very similar and very small in each compound, because the two groups lie far away in terms of covalent bonds. Our results provide another proof that the pair-interactivity parameter is a relatively invariant quantity and is influenced to a lesser extent by electron withdrawing effects of other sites than the microscopic protonation constants.^[23]

Conclusions

Modification of morphine at C-3 position changes not only the analgesic activity of the molecule, but also gives the opportunity for further reactions. The 3-OH derivatives (morphine, oxymorphone, naloxone, naltrexone) containing a free carboxy group are haptens by definition. Although the functionalization of the C-3 hydroxy group leads to decreased analgesic effects and the 3-O-morphine haptens are not suitable for vaccine against drug abuse, these derivatives are excellent candidates for selective determination of morphine alkaloids in body fluids, using various immunoassays (radioimmunoassay, enzyme immunoassay).

Treating the phenolic hydroxy group containing morphine derivatives with ethyl bromo- or chloroacetate led us to the intermediate 3-O-functionalized esters. Hydrolyzing them in alkaline solution and adjusting the pH with hydrochloric acid yielded the desired HCl salts of 3-O-haptens, namely 2-(morphin-3-O-yl)acetic acid, 2-(oxymorphon-3-O-yl)acetic acid, 2-(naloxon-3-O-yl)acetic acid and 2-(naltrexon-3-O-yl)acetic acid. Previously only ethyl 2-(morphin-3-O-yl)acetic acid (3-O-carboxymethylmorphine ethyl ester) and 2-(morphin-3-O-yl)acetic acid HCl were synthesized, but not fully characterized, all of the other

compounds, to the best of our knowledge are synthesized and characterized/described for the first time (including the aforementioned two molecules, too). In addition, these carboxy group containing derivatives show structural similarity to the important metabolite morphine-3-glucuronide. Thus, the protonation macro- and microconstants of these novel compounds were determined, too.

Experimental Section

General Information

The reagents and indicator molecules were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Alfa Aesar (Haverhill, MA, USA) and used without further purification. Solvents were freshly distilled prior to use and were dried over anhydrous Na_2SO_4 . ^1H - and ^{13}C -NMR spectra were recorded on a 600-MHz Varian VNMRs spectrometer (Varian, Inc., NMR Systems, Palo Alto, CA, USA, Varian is now part of Agilent Technologies) in (D_6)DMSO or D_2O solutions; δ is given in ppm relative to (D_6)DMSO and DSS as internal standard, retrospective. ^1H - and ^{13}C -NMR signals were assigned on the basis of one- and two-dimensional hetero-nuclear experiments (gHMBCAD and gHSQCAD). Melting points were taken on a Stuart SMP-3 apparatus (Global Science NZ Ltd., Auckland, New Zealand). The high-resolution accurate masses were determined with a Dionex Ultimate 3000 UHPLC system hyphenated with an Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (ESI) (Thermo Fischer Scientific, Waltham, MA, USA). IR spectra were recorded with Perkin Elmer FT-IT Spectrometer, Spectrum 2000 Diode Pumped Nd:YAG Laser. To determine the molar ellipticity Jasco J-720 Spectropolarimeter (Jasco Corp., Tokyo, Japan) was used (range 170–800 nm, light source: 450 W xenon lamp, water cool) with 50 nm/min record, 1 s response and 3 times accumulation. Reaction progress was observed by thin-layer chromatography on commercial silica gel plates (Merck silica gel F254 on aluminum sheets, Darmstadt, Germany), by using different mobile phases. For column chromatography, Kieselgel 60 (particle size 0.040–0.063 mm, ordered from VWR Chemicals, Radnor, PA, USA) was employed. The indicator molecules for the NMR-pH titrations (sodium acetate, chloroacetic acid and dichloroacetic acid) were from Sigma-Aldrich, the deuterated solvent D_2O from VWR International. Other chemicals of analytical grade were obtained from commercial suppliers and used without further

purification. Bidistilled water was used for all solutions.

NMR-pH Titrations

All NMR measurements were performed on a Varian VNMRs spectrometer (600 MHz for ^1H). Spectra were recorded at 25 °C. The water signal was suppressed by presaturation.^[25] Spectra were processed using VNMRj 3.2a software. Initial pH values were read on a Metrohm 2.780.0010 precision pH meter with a 6.0258.600 Unitrode glass Pt 1000 electrode (Metrohm AG, Herisau, Switzerland). For the analysis of NMR titration curves of proton chemical shifts versus pH, the software Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) was used.^[26] Titrations were carried out the same way as in our previous publication.^[19]

pH-Potentiometric Titrations

A 716 DMS Titrino automatic titrator (Metrohm) with a Metrohm 6.0234.110 combined pH glass electrode was used for the potentiometric titrations, under automatic PC (personal computer) control. Non-linear parameter fitting with Origin 8 provided the protonation constants from the volume differences.^[27] The method of measurements was identical to our previous work.^[19]

3-O-hapten Ester Synthesis

Ethyl 2-(morphin-3-O-yl)acetate (**5**): Metallic sodium (0.16 g, 6.96 mmol) was dissolved in absolute ethanol (30 mL), morphine hydrochloride salt (1.00 g, 3.11 mmol) was added to the solution and treated with ethyl bromoacetate (0.41 mL, 3.73 mmol). The mixture was stirred and refluxed for 6 h. It was monitored by TLC. After conversion, the inorganic salts were filtered and the solvent was evaporated. The crude product was purified by column chromatography using chloroform/methanol 9:1. Yield: 62%

Ethyl 2-(oxymorphon-3-O-yl)acetate (**6**): Oxymorphone base (0.50 g, 1.66 mmol) was dissolved in acetone (30 mL). In the presence of potassium carbonate (0.37 g, 2.68 mmol) and potassium iodide (0.40 g, 2.41 mmol) the mixture was treated with ethyl chloroacetate (0.25 mL, 2.34 mmol). After stirring and refluxing for 12 h the solvent was evaporated and the residue was suspended with brine. Then it was extracted 2 times with chloroform

(25 mL), the organic layers were unified and dried over sodium sulfate. On TLC only one spot was observed (chloroform/acetone/diethylamine 5:4:1). The salts were filtered and the chloroform was evaporated. Yield: 70%

Ethyl 2-(naloxon-3-O-yl)acetate (**7**): Naloxone base (0.80 g, 2.44 mmol) was dissolved in acetone (25 mL). In the presence of potassium carbonate (0.42 g, 3.04 mmol) and potassium iodide (0.50 g, 3.01 mmol), the mixture was treated with ethyl chloroacetate (0.32 mL, 3.00 mmol). After stirring and refluxing for 12 h, the inorganic salts were filtered and the solvent was evaporated. The crude product was purified with column chromatography by using chloroform/methanol 9:1. Yield: 67%

Ethyl 2-(naltrexon-3-O-yl)acetate (**8**): Naltrexone base (0.34 g, 1.00 mmol) was dissolved in acetone (20 mL) and the solution was treated with ethyl bromoacetate (0.13 mL, 1.20 mmol) in the presence of potassium carbonate (0.69 g, 5.00 mmol). The mixture was stirred and refluxed for 12 h. The conversion was followed by TLC. The inorganic salts were filtered and the solvent was evaporated. The crude product was purified with column chromatography by using chloroform/methanol 9:1. Yield: 83%

3-O-hapten Ester Hydrolysis

Every ester was hydrolyzed by following the same general procedure. The esters were dissolved in a minimal amount of ethanol and the solutions were stirred with 0.1 M sodium hydroxide for 30–60 min at 60 °C. The pH was set to 2–3 with diluted hydrochloric acid and the solvent was evaporated. In every case the 3-O-hapten hydrochloride salts were obtained.

2-(Morphin-3-O-yl)acetic acid HCl (**9**): (**5**) (0.13 g, 0.35 mmol) was dissolved in ethanol (0.1–0.2 mL) and treated with sodium hydroxide solution (3.50 mL). Yield: 70%

2-(Oxymorphon-3-O-yl)acetic acid HCl (**10**): (**6**) (0.10 g, 0.26 mmol) was dissolved in ethanol (0.1–0.2 mL) and treated with sodium hydroxide solution (2.60 mL). Yield: 97%

2-(Naloxon-3-O-yl)acetic acid HCl (**11**): (**7**) (0.14 g, 0.33 mmol) was dissolved in ethanol (0.1–0.2 mL) and

treated with sodium hydroxide solution (3.30 mL). Yield: 80%.

2-(Naltrexon-3-O-yl)acetic acid HCl (**12**): (**8**) (0.10 g, 0.24 mmol) was dissolved in ethanol (0.1–0.2 mL) and treated with sodium hydroxide solution (2.40 mL). Yield: 98%

Acknowledgements

This work was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (G.T.). The financial support from Bolyai + New National Excellence Program (UNKP-20-5) of the Ministry for Innovation and Technology is highly appreciated (G.T.).

Author Contribution Statement

The manuscript was written with the contributions of all authors. IK and SH were responsible for the synthesis, purification and NMR and IR measurements and assignments of the molecules. KM and BT determined the protonation constants of the compounds. GT was responsible for HR-MS measurements. EK was responsible for the CD measurements and PH for the interpretation of the CD results. SH and IK conceived and designed the project. All authors discussed the results and commented on the manuscript. All authors have given approval to the final version of the manuscript.

References

- [1] S. Spector, C. Parker, 'Opioid Alkaloid Specific Antibodies', U. S. Patent 3, 822, 245, 2 July 1974.
- [2] S. Spector, C. W. Parker, 'Morphine: Radioimmunoassay', *Science* **1970**, *168*, 1347–1348.
- [3] K. E. Rubinstein, E. F. Ullman, 'Inhibitable Enzyme Amplification Assay', U. S. Patent 4, 203, 802, 20 May 1980.
- [4] K. F. Buechler, 'Opiate Derivatives and Protein and Polypeptide Opiate Derivative Conjugates and Labels', U. S. Patent 5, 610, 283, 11 March 1997.
- [5] D. F. Heiman, D. S. Raden, R. E. Dubler, 'Immunoassay for Opiate Alkaloids and Their Metabolites, Tracers, Immunogens and Antibodies', U. S. Patent 4, 939, 264, 14 July 1986.
- [6] S. Gandhi, P. Sharma, N. Capalash, R. S. Verma, C. R. Suri, 'Group-selective antibodies based fluorescence immunoassay for monitoring opiate drugs', *Anal. Bioanal. Chem.* **2008**, *392*, 215–222.
- [7] V. R. Spiehler, D. Reed, R. H. Cravey, W. P. Wilcox, R. F. Shaw, S. Holland, 'Comparison of results for quantitative determination of morphine by radioimmunoassay, enzyme immunoassay, and spectrofluorometry', *J. Forensic Sci.* **1975**, *20*, 647–655.
- [8] S. Spector, 'Distribution of drugs by radioimmunoassay', *Pharmacol. Rev.* **1982**, *34*, 73–75.
- [9] J. W. A. Findlay, 'Applications of immunoassay methods to drug disposition studies', *Drug Metab. Dispos.* **1987**, *18*, 83–129.
- [10] C. W. Hand, R. A. Moore, H. C. McQuay, M. C. Allen, J. W. Sear, 'Analysis of morphine and its major metabolites by differential radioimmunoassay', *Anal. Biochem.* **1987**, *24*, 153–160.
- [11] M. E. Olson, L. M. Eubanks, K. D. Janda, 'Chemical Interventions for the Opioid Crisis: Key Advances and Remaining Challenges', *J. Am. Chem. Soc.* **2019**, *141*, 1798–1806.
- [12] B. Kinsey, 'Vaccines against drugs of abuse: Where are we now?', *Adv. Vaccines* **2014**, *2*, 106–117.
- [13] O. Torres, C. Alving, A. Jacobson, K. Rice, G. Matyas, 'Practical Considerations for the Development of Vaccines Against Drugs of Abuse', In *Biologics to Treat Substance Use Disorders: Vaccines, Monoclonal Antibodies, and Enzymes*, 1st ed.; Montoya, I. D., Ed.; Springer International Publishing: Cham, Switzerland, 2016; pp. 397–424.
- [14] P. T. Bremer, J. E. Schlosburg, M. L. Banks, F. F. Steele, B. Zhou, J. L. Poklis, K. D. Janda, 'Development of a Clinically Viable Heroin Vaccine', *J. Am. Chem. Soc.* **2017**, *139*, 8601–8611.
- [15] F. Li, K. Cheng, J. F. G. Antoline, M. R. Iyer, G. R. Matyas, O. B. Torres, R. Jalah, Z. Beck, C. R. Alving, D. A. Parrish, et al., 'Synthesis and immunological effects of heroin vaccines', *Org. Biomol. Chem.* **2014**, *12*, 7211–7232.
- [16] G. Matyas, A. V. Mayorov, K. C. Rice, A. E. Jacobson, K. Cheng, M. R. Iyer, F. Li, Z. Beck, K. D. Janda, C. R. Alving, 'Liposomes containing monophosphoryl lipid A: A potent adjuvant system for inducing antibodies to heroin hapten analogs', *Vaccine* **2013**, *31*, 2802–2810.
- [17] J. W. A. Findlay, R. F. Butz, E. C. Jones, 'Relationships between immunogen structure and antisera specificity in the narcotic alkaloid series', *Clin. Chem.* **1984**, *27*, 1524–1535.
- [18] M. Koida, M. Takahashi, H. Kaneto, 'The morphine-3-glucuronide directed antibody: Its immunological specificity and possible use for radioimmunoassay of morphine in urine', *Japan J. Pharmacol.* **1974**, *24*, 707–714.
- [19] I. Köteles, K. Mazák, G. Tóth, B. Túz, S. Hosztafi, 'Synthesis of Potential Haptens with Morphine Skeleton and Determination of Protonation Constants', *Molecules* **2020**, *25*, 4009.
- [20] A. T. Crone, N. Purdle, 'Circular Dichroism Spectra of Opium Alkaloids in Aqueous Media', *Anal. Chem.* **1981**, *53*, 17–21.
- [21] G. Snatzke, M. Kajtár, F. Werner-Zamoiska, 'Influence of substitution pattern on the benzene ¹L_b band cotton effect', *Tetrahedron* **1972**, *28*, 281–288.
- [22] K. Mazák, B. Noszá, S. Hosztafi, 'Advances in the physicochemical profiling of opioid compounds of therapeutic interest', *ChemistryOpen* **2019**, *8*, 879–887.
- [23] K. Mazák, B. Noszá, 'Advances in microspeciation of drugs and biomolecules: species-specific concentrations, acid-base properties and related parameters' *J. Pharm. Biomed. Anal.* **2016**, *130*, 390–403.
- [24] A. Avdeef, B. Testa, 'Physicochemical profiling in drug research: a brief survey of the state-of-the-art of exper-

- imental techniques', *Cell. Mol. Life Sci.* **2002**, *59*, 1681–1689.
- [25] M. Somlyay, G. Orgován, B. Noszál, 'The site-specific protonation constants of spectinomycin, characterized by ^1H and ^{15}N NMR methods', *Curr. Pharm. Anal.* **2015**, *11*, 4–10.
- [26] G. Jakab, D. Bogdán, K. Mazák, R. Deme, Z. Mucsi, I. Mándity, B. Noszál, N. Kállai-Szabó, I. Antal, 'Physicochemical profiling of baicalin along with the development and characterization of cyclodextrin inclusion complexes', *AAPS PharmSciTech* **2019**, *20*, 314.
- [27] K. Mazák, S. Hosztafi, M. Kraszni, B. Noszál, 'Physicochemical profiling of semisynthetic opioids' *J. Pharm. Biomed. Anal.* **2017**, *135*, 97–105.

Received February 23, 2021

Accepted May 18, 2021