Low Molecular Weight Proteins as Carriers for Renal Drug Targeting. Preparation of Drug-Protein Conjugates and Drug-Spacer Derivatives and Their Catabolism in Renal Cortex Homogenates and Lysosomal Lysates†

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Low molecular weight proteins (LMWPs) are known to be reabsorbed and catabolized primarily by the proximal tubular cells of the kidneys. As such, LMWPs might serve as drug carriers that release drugs site-specifically in the kidney. We tested this concept in vitro by coupling different drugs to the LMWP lysozyme both directly (amide bond) and via different spacers: oligopeptides (amide bond), (poly-)α-hydroxy acids (ester bond), and a pH sensitive *cis*-aconityl spacer (amide bond). The capability of the kidney to release the parent drug from such drug-spacer derivatives and drug-LMWP conjugates by enzymatic or chemical hydrolysis of the bond was tested by incubation experiments in renal cortex homogenates and lysosomal lysates. Directly coupled conjugates of terminal carboxyl group containing drugs and lysozyme were catabolized to single amino acids, but did not result in release of the parent drug. The amide bond between the drug and the final amino acid (lysine) appeared to be stable in the incubation milieu. Different oligopeptide spacers coupled to the drugs showed similar results: the oligopeptide itself was cleaved but the amide bond between the drug and different single amino acids remained untouched. Only amide bonds of derivatives of carboxylic drugs with peptide structures themselves were cleaved. Some of the directly coupled conjugates of terminal amino drugs and oligopeptides showed clear release of the parent drug whereas others were stable. Terminal amino drugs were rapidly released from an acid-sensitive *cis*-aconityl spacer. Terminal carboxyl group containing drugs were enzymatically released from their glycolic and lactic ester spacers at different rates. These kinds of drugs were also released as parent drug from LMWP conjugates with ester spacers like L-lactic acid. Increasing spacer length by intercalating a tetra(L-lactic acid) molecule between the drug and the protein further increased the extent and rate of drug release, indicating increased accessability of the bond to the enzymes. Terminal amino group containing drugs were rapidly generated as parent drug from LMWP conjugates using an acid-sensitive spacer. In addition the conjugates were found to be adequately stable in plasma, considering their rapid clearance from the bloodstream. It is concluded that LMWPs may indeed be of use as carriers for specific renal delivery of drugs, since renal cortex homogenates and lysosomal lysates are able to catabolize the protein and generate the parent drug from drug-LMWP conjugates bearing suitable spacers. The option of enzymatic release is limited by the narrow specificity of the lysosomal enzymes. This has profound implications for the synthesis of suitable conjugates, since the nature of the drug itself, the type of bond, and also spacer length largely determine whether release of the parent drug will occur. Tailor-made spacers containing the correct mode of attachment and the right spacer length are required for this option. Chemical hydrolysis, using acid-sensitive linkers, is suggested as a viable alternative approach.

Introduction

The kidney is an especially suitable organ for drug targeting. Many therapeutic indications are often the result of chronic therapies with various drugs, examples being inflammation and urinary tract infections. Dose regimens of many of these drugs are accompanied by unwanted side effects.[1] The kidney itself is a well-perfused organ that is involved in the metabolism of many endogeneous and exogeneous compounds. One might expect that prodrug strategies could increase therapeutic indices of different drugs through selective transport to the kidney, followed by the generation of parent active drug.[2]

Various prodrug approaches have been described on the basis of more or less kidney-specific enzymes. Examples are γ-glutamyltransferase (prodrugs: γ-glutamyldopamide,[3] γ-glutamylsulfamethoxazole[4,5]), and phosphatase (prodrug: *N*-methyldopamine 4-*O*-phosphate[6]). These

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† Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9.). All amino acids have the L (*S* in Cahn-Ingold-Prelog system) configuration unless otherwise noted. Other abbreviations used are as follows: ACN, acetonitrile; HAc, acetic acid; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DCC, dicyclohexylcarbodiimide; ECDI, ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Boc, *tert*-butyloxycarbonyl; NHS, *N*-hydroxysuccinimide; S-NHS, *N*-hydroxysulfosuccinimide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; Nap, naproxen; Indo, indomethacin; B, benzoic acid; LYSO, lysozyme; TEA, triethylamine; BSA, bovine serum albumin, TRIS, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran; PMB, pentamethylbenzyl; 4-DMAP, 4-(dimethylamino)pyridine; SM, sulfamethoxazole; aco-SM, *N*-*cis*-aconitylsulfamethoxazole; SM-aco-LYSO, *N*-*cis*-aconitylsulfamethoxazole linked to egg-white lysozyme (1:1); PBS, phosphate-buffered saline.

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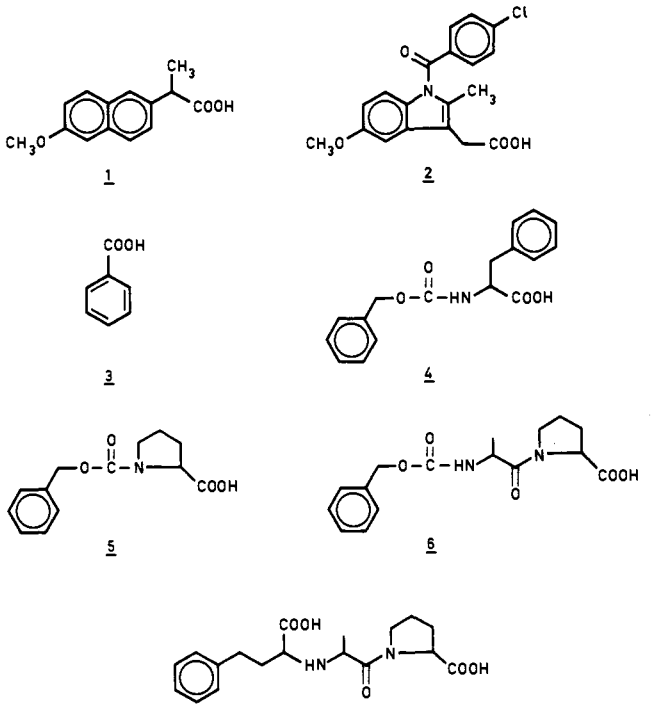
Chart I. Model Compounds and Model Drugs with a Carboxyl Group[a]



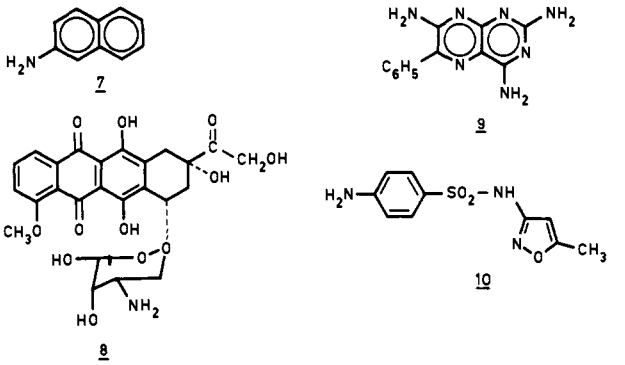
[a] Model drugs are 1, naproxen; 2, indomethacin. Model compounds are 3, benzoic acid; 4, Z-Phe; 5, Z-Pro; Z-Ala-Pro. The unlabeled structure represents enalaprilate.

enzymes are presumed to mediate drug generation in the kidney by cleavage of the prodrug moiety attached to the drug. However, kidney accumulation may be low due to binding to plasma proteins or limited transport to the kidney.[5] Low molecular weight proteins (LMWP's) have attractive properties as potential renal selective and biodegradable drug carriers. These proteins are reported to accumulate extensively in the proximal tubule cells of the kidney and are subsequently hydrolyzed into their single amino acid constituents in the lysosomes of these cells.[7,8] Furthermore, these proteins offer opportunities for covalent drug attachment owing to the presence of different functional reactive groups. For these reasons we decided to explore the potential of these proteins for renal drug targeting. As LMWP egg-white lysozyme (MW 14400 and isoelectric point of 11) was chosen. Lysozyme contains 149 amino acids and seven free amino groups. These groups are available for drug derivatization.

For the synthesis of a suitable, kidney selective, drug–protein conjugate it is essential that the bond that links drug and carrier will be degradable; otherwise the active parent drug will not be generated. The lysosomal medium offers in principle several possibilities to cleave such bonds, either enzymatically or chemically. The lysosomes contain many different hydrolytic enzymes such as proteinases and esterases. The pH of the lysosomes is 4–5.[9]

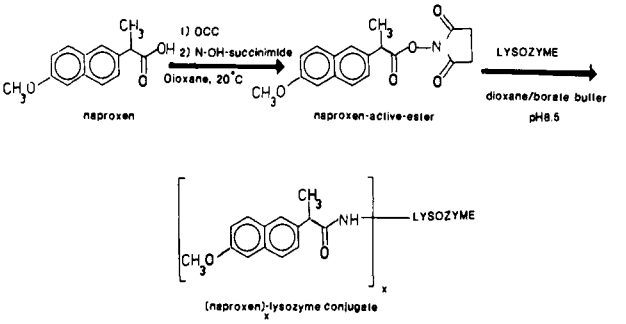
In some cases it may be possible to couple the drug directly to the carrier.[10,11] However, for other drugs

Chart II. Model Compounds and Model Drugs with a Primary Amino Group[a]



[a] Model compound 7 is β-naphthylamine. Model drugs are 8, adriamycin; 9, triamterene; 10, sulfamethoxazole.

Scheme I. The *N*-Hydroxysuccinimide Reaction of Naproxen and the LMWP Lysozyme[a]



[a] Symbol *x* denotes various degrees of drug substitution.

suitable biodegradable spacers between drugs and carrier are reported to be essential for drug generation.[12,13] The nature of both drug and carrier, as well as the metabolic capacity in a specific target-cell, are factors which will determine the specific type of spacer.

The purpose of the present study is to inventory different drug–LMWP conjugates with regard to their capacity to act as suitable substrates for renal catabolism and parent-drug release. Various model compounds were selected on the basis of their coupling potential with proteins or spacers, as well as their potential biodegradability. These model compounds included both drugs with a terminal carboxylic group and drugs with a terminal amino group (Charts I and II). As model bonds we examined a direct amide linkage to the protein (carboxylic group attached to the ε-amino group of lysine), an indirect amide bond via oligopeptide spacers, ester bonds, and an acid-labile amide bond. Amide and ester derivatives with spacer

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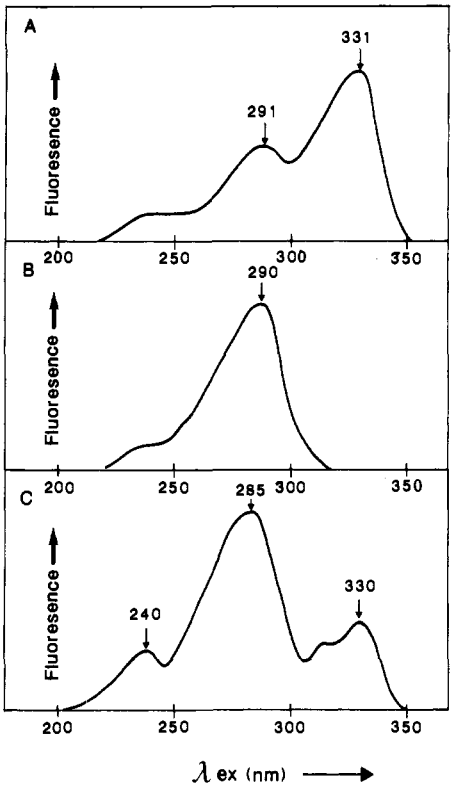

Figure 1. Excitation-fluorescence spectra, measured at an emission wavelength of 360 nm, of (panel A) naproxen, (panel B) lysozyme, and (panel C) $(naproxen)_x$-lysozyme. The degree of drug substitution can be measured at excitation wavelength 330 nm and emission wavelength 360 nm.

candidates, mainly natural L-amino acids or oligopeptides, were synthesized. Since these products may be regarded as potential catabolytes in the metabolism of a drug–protein conjugate, these compounds were studied with respect to parent active drug generation in renal cortex homogenates and lysosomal lysates. Thereafter cleavable drug–spacer derivatives could be selected for further coupling to the protein. These conjugates were also tested in these systems.

Results

Preparation and Characterization of Conjugates and Model Drug Derivatives. Conjugates and derivatives of the model compounds and model drugs are listed in Tables I and II. The model drug naproxen (1, Chart I) was coupled to the LMWP lysozyme by reaction of the carboxyl group with the free amino functionalities of the protein. (Derivatives of 1 and other compounds are indicated further in the text and tables by a letter following the number of the parent compound). Coupling procedures examined included a water-soluble-carbodiimide method using ECDI by the method of Davis[14] and reactions of the acid chloride derivative and *N*-hydroxy- and *N*-hydroxysulfosuccinimide derivatives of 1. The *N*-hydroxysuccinimide (NHS) method was found to be superior. Criteria were the degree of substitution, yield of soluble protein conjugate, coupling efficiency, and protein cross-linking. For the NHS coupling procedure[15] (Scheme

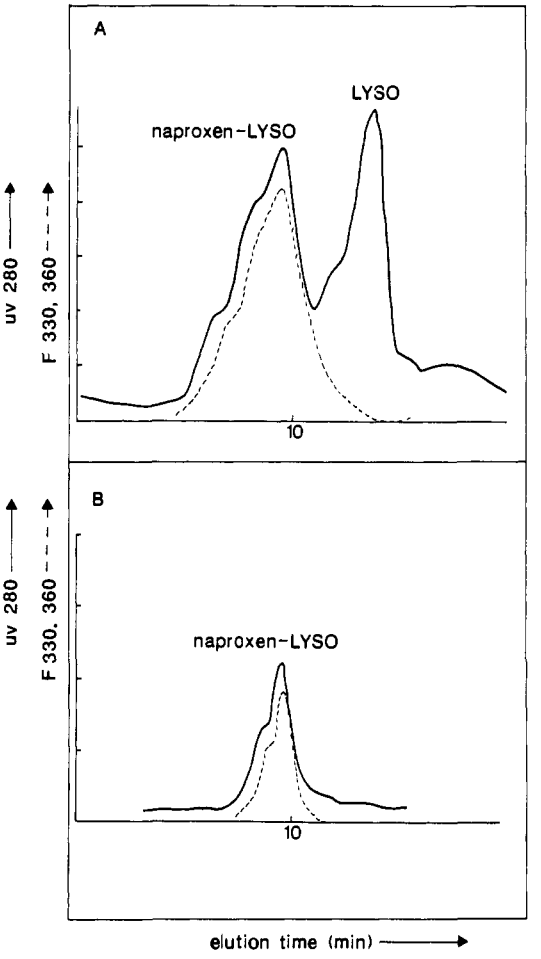

Figure 2. Separation of naproxen–lysozyme (1:1) from unreacted native lysozyme by FPLC-cationic exchange chromatography. Naproxen is monitored by specific fluorescence detection; lysozyme is monitored by UV detection (A_{280}).

I) optimal conditions were determined by varying molar ratios and concentrations of reactants, composition of coupling buffer [i.e. ratio of organic component (dioxane) and aqueous component (borate buffer)], and pH of the coupling buffer. The influence of the nature of the protein was investigated by also coupling 1 to bovine serum albumin. The optimal solvent system was a 1,4-dioxane/borate buffer, pH 8.5 (20/80). Variation of the molar ratios of reactants resulted in conjugates with different molar drug:protein ratios. Up to six molecules of 1 to one molecule lysozyme could be incorporated. The yield of soluble lysozyme conjugates appeared to be limited by precipitation of highly substituted products. Coupling of 1 to BSA resulted in products with a molar coupling ratio of up to 18. The yields of soluble albumin conjugates were more than 95% (Table III).

Peptide spacers were intercalated by initially linking the drug to the oligopeptide spacer (**1b–e**). The drug–peptide derivative was then coupled to the protein by the *N*-hydroxysuccinimide procedure whereby the terminal carboxylic acid group is linked to the amino groups of the protein. This is illustrated for the ethyl ester of L-leucine in Scheme II. Unreacted or noncovalently bound drug(–spacer) was removed by dialysis, ultrafiltration, and gel-exclusion chromatography. Unreacted lysozyme could be removed by a subsequent cationic-exchange separation procedure. Examples of the fluorescence monitoring and FPLC separations are given in Figures 1 and 2. Separation was possible on the basis of the slight charge difference between conjugated and native lysozyme. Amide forma-

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Table I. In Vitro Cleavage Data for Terminal Carboxyl Group Containing Model Compounds and Model Drugs

compound		synthesis and HPLC analysis		% drug release after 24-h incubation[c]							observed
no.	name	S no.[a]	HPLC system, t_R[a] (min)	% yield	pH 5	pH 7.4	P	H5	H7.4	L5	L7.4	catabolyte[d]
1	naproxen (Nap)		Ia, 11.8 Ib, 9.6 II, 25.8		S	S	S	S	S	S	S	
1a	Nap-LYSO	S7		60	0	0	0	0	0	0	0	ϵ-Nap-Lys
1b	Nap-Leu-LYSO	S7		63	<1	<1	<1	<1	<1	<1	<1	Nap-Leu
1c	Nap-Leu-Gly-Gly-LYSO	S7		65	<1	<1	<1	<1	<1	<1	<1	Nap-Leu
1d	Nap-Tyr-LYSO	S7		60	<1	<1	<1	<1	<1	<1	<1	Nap-Tyr
1e	Nap-Tyr-Gly-Gly-LYSO	S7		63	<1	<1	<1	<1	<1	<1	<1	Nap-Tyr
1f	ϵ-Nap-Lys		Ia, 3.3	60	0	0	0	0	0	0	0	ϵ-Nap-Lys
1g	Nap-Gly	S1	Ia, 5.8	93	0	0		0	0			Nap-Gly
1h	Nap-Val	S1	Ia, 16.6	84	0	0		0	0			Nap-Val
1i	Nap-Leu	S1	Ia, 17.8	80	0	0		0	0			Nap-Leu
1j	Nap-Phe	S1	Ia, 20.6	72	0	0		0	0			Nap-Phe
1k	Nap-Tyr	S1	Ia, 8.4 II, 20.2	67	0	0		0	0			Nap-Tyr
1l	Nap-Trp	S1	Ia, 19.6	68	0	0		0	0			Nap-Trp
1m	Nap-Glu	S1	Ia, 8.6	80	0	0		0	0			Nap-Glu
1n	Nap-Arg[e]	S1	Ia, 4.1	100	0	0		0	0			Nap-Arg
1o	Nap-Leu-Gly-Gly	S1		80	0	0		0	0			Nap-Leu
1p	Nap-Tyr-Gly-Gly	S1		75	0	0		0	0			Nap-Tyr
1q	Nap-glycolic acid (E)	S2	Ib, 11.8	85	1	2	8	81	8	45	5	Nap
1r	Nap-L-lactic acid (E)	S2	Ib, 16.2	90	0.6	1.4	13	100	15	100	3	Nap
1s	Nap-L-lact-LYSO (E)	S7		60	1.1	6.9	6.8	2.0		2.0		Nap
1t	Nap-(L-lact)$_4$-LYSO (E)	S7		10	2			11.0		12.0		Nap
2	indomethacin (Indo)		III, 10.8 IV, 5.2		S	S	S	S	S			
2a	ϵ-Indo-α-Z-Lys	S3	III, 85.0 IV, 10.6	78	0	0		0	0			ϵ-Indo-α-Z-Lys
2b	Indo-Gly	S3	IV, 4.6	70	0	0		0	0			Indo-Gly
2c	Indo-Leu	S3	III, 27.6 IV, 7.0	80	0	0		0	0			Indo-Leu
2d	Indo-Phe	S3	III, 35.0 IV, 7.8	80	0	0		0	0			Indo-Phe
2e	Indo-Tyr	S4	IV, 5.0	41	0	0		0	0			Indo-Tyr
2f	Indo-Trp	S3	III, 35.8 IV, 7.4	81	0	0		0	0			Indo-Trp
2g	Indo-Glu	S3	III, 3.8 IV, 3.0		0	0		0	0			Indo-Glu
2h	Indo-Arg	S3-5		0								
3	benzoic acid (B)		V, 3.8 VI, 3.6 VIIa, 3.0		S	S	S	S	S			
3a	ϵ-B-α-Z-Lys	S6	VI, 10.1	89	0	0		0	0			ϵ-B-α-Z-Lys
3b	B-Gly	S6	V, 1.7 VI, 2.1 VIIa, 4.8	97	0	0		0	0			B-Gly
3c	B-Leu	S6	VI, 6.3	93	0	0		0	0			B-Leu
3d	B-Phe	S6	VI, 7.0 VIIa, 12.7	92	0	0		0	0			B-Phe
3e	B-Tyr	S6	VI, 3.0 VIIa, 4.9	62	0	0		0	0			B-Tyr
3f	B-Trp	S6	VI, 7.1 II, 6.5	81	0	0		0	0			B-Trp
3g	B-Glu	S6	VI, 2.1 VIIa, 2.2	97	0	0		0	0			B-Glu
3h	B-Arg	S6	VI, 2.3	93	0	0		0	0			B-Arg
4	Z-Phe		I, 8.2		S	S		S	S	S	S	
4a	Z-Phe-Ala		I, 9.2		0	0		100	100	100	100	Z-Phe
5	Z-Pro		I, 5.0		S	S		S	S	S	S	
5a	Z-Pro-Phe		I, 9.2		0	0		100	100	100	100	Z-Pro
6	Z-Ala-Pro		I, 6.6		S	S		S	S	S	S	
6a	Z-Ala-Pro-Phe		I, 11.6	100	0	0		100	96			Z-Ala-Pro

[a] S no. refers to synthesis number as described in the Experimental Section. [b] The Roman numerals refers to the HPLC system as described in the Experimental Section; t_R refers to the retention time of the compound. [c] Drug release is expressed as the percentage of total present drug after 24 h of incubation; the values represent the mean of at least two separate incubations. Incubation media were pH 5.0 buffer (pH 5), pH 7.4 buffer (pH 7.4), plasma buffered at pH 7.4 (P), cortex homogenate at pH 5.0 and 7.4, respectively (H5 and H7.4) and lysosomal lysates at pH 5 and 7.4, respectively (L5 and L7.4) (see Experimental Section). [d] Observed catabolyte refers to the ultimate catabolyte as observed during incubation with lysosomal lysates. Symbols in the table: S = stable (i.e. the drug itself is not degraded or converted into other metabolites); (E) denotes ester bond. [e] There is no absolute evidence that coupling occurred exclusively at the α-amino group. Owing to the lack of parent drug release, this point was not explored further.

tion between a compound containing a carboxyl group and a protein resulted in a reduction of net positive charge of the protein, since the drug neutralizes the positive charge on the ϵ-amino groups of the lysine amino acids in the

Table II. In Vitro Cleavage Data for Primary Amino Group Containing Model Compounds and Model Drugs

compound		synthesis and HPLC analysis			% drug release after 24-h incubation[c]							observed
no.	name	S no.[a]	HPLC system, t_R (min)[b]	% yield	pH 5	pH 7.4	P	H5	H7.4	L5	L7.4	catabolyte[d]
7	β-naphthylamine (β-naph)				S	S		S	S	S	S	
7a	Leu-β-naph				0	0		100	100	100	100	β-naph
7b	Gly-Phe-β-naph				0	0		100	100	100	100	β-naph
8	adriamycin (adria)		XI, 6.4		S	S	S	S	S	S	S	
8a	Leu-adria		Xi, 9.8		1	3	37	55	45	9	58	adria
8b	Gly-Leu-adria		XI, 11.6		0	0	16	34	47	6	36	adria Leu-adria
9	triamterene (t)		VIIb, 5.0		S	S		S	S			
9a-I	Leu-t		VIIb, 28.4	9	0	0		<5	<5			Leu-t
9a-II	Leu-t		VIIb, 31.2	31	0	0		<5	<5			Leu-t
9a-III	Leu-t		VIIb, 35.8	6	0	0		0	0			Leu-t
10	sulfamethoxazole (SM)		X, 6.1		S	S	S	S	S	S	S	
10a	*cis*-aco-SM		X, 4.6	100	100	15						SM
10b	SM-aco-LYSO	S7			80	7	7	84	8	84	8	SM

[a] S no. refers to synthesis number as described in the Experimental Section. [b] The Roman numerals refers to the HPLC system as described in the Experimental Section; t_R refers to the retention time of the compound. [c] Drug release is expressed as the percentage of total present drug after 24 h of incubation; the values represent the mean of at least two separate incubations. Incubation media were pH 5, C buffer (pH 5), pH 7.4 buffer (pH 7.4), plasma buffered at pH 7.4 (P), cortex homogenate at pH 5.0 and 7.4, respectively (H5 and H7.4), and lysosomal lysates at pH 5 and 7.4 (L5 and L7.4), respectively (see Experimental Section). [d] Observed catabolyte refers to the ultimate catabolyte as observed during incubation with lysosomal lysates. Symbols in the table: S = stable (i.e. the drug itself is not degraded or converted into other metabolites); (E) denotes ester bond.

Table III. Coupling of Naproxen to Lysozyme and BSA

egg-white lysozyme			bovine serum albumin		
molar ratio reactants[a]	coupling ratio[b]	% yield[c]	molar ratio reactants[a]	coupling ratio[b]	% yield[c]
0:1	0.0:1	95	0:1	0.0:1	98
1:1	0.5:1	38	1:1	1.0:1	98
5:1	2.2:1	16	10:1	3.0:1	96
10:1	6.3:1	<4	100:1	18.5:1	97
20:1	4.4:1	<4			

[a] Molar ratio of NHS ester of naproxen:protein. [b] Molar ratio of drug:protein, determined by quantitative analysis of both drug and protein (see Experimental Section). No noncovalently bound drug (<0.2%) was present as determined by HPLC. [c] Yield refers to recovered soluble drug-protein conjugate after dialysis and lyophilization.

protein. The differences in net charge between conjugated and native lysozyme resulted in different FPLC elution profiles. In this respect, soluble 1-lysozyme (1:1) could be obtained by the removal of unreacted lysozyme from a 0.5 molar mixture by this method of chromatography.

The degree of substitution of the conjugates was determined by analysis of drug, protein, and spacer present [values either expressed as g/g (%) or molar coupling ratio]. Drug content was assessed by HPLC, selective fluorimetric measurements, or, indirectly, determination of unreacted amino groups of the protein. A comparison of these independent assays of drug substitution is shown in Table IV.

The HPLC method can discriminate between covalently and noncovalently bound drug. Total bound drug is determined after an initial alkaline hydrolytic treatment that results in cleavage of the amide bond and liberation of 1. Noncovalently bound drug was assayed immediately after drug extraction. The amount of noncovalently bound drug present in the conjugates was less than 0.2%. A quantitative fluorimetric measurement was possible, since the selective molar fluorescence of 1 at 330 and 360 nm (excitation and emission wavelength, respectively) (Figure 1) was not significantly altered as was established also by linking 1 to a single amino acid resulting in ε-naproxen-lysine (1f). Furthermore, free unreacted amino groups of

Scheme II. Reaction Scheme for the Intercalation of Leucine as a Spacer between Naproxen and Lysozyme (cp. 1b)

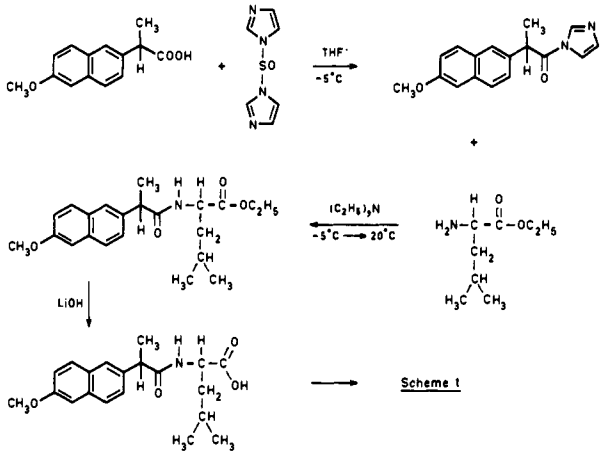

Table IV. Degree of Substitution of Naproxen-Lysozyme Conjugates

		degree of substitution[a]					
		molar ratio of drug:protein				drug content (%, g/g)	
product no.	product	HPLC	F	H	mean	covalently bound	noncovalently bound
1a-I	$Nap_{0.5}$-LYSO	0.48 ± 0.049:1	0.62 ± 0.003:1	0.5 ± 0.16:1	0.5	0.76 (100)	0.001 (0.13)
1a-II	$Nap_{1.4}$-LYSO	1.40 ± 0.014:1	1.19 ± 0.021:1	1.5 ± 0.14:1	1.4	2.24 (100)	0.003 (0.13)
1a-III	$Nap_{2.0}$-LYSO	2.11 ± 0.033:1	2.0 ± 0.14:1	2.0 ± 0.20:1	2.0	3.37 (100)	0.005 (0.15)

[a] Degree of substitution is presented as the molar ratio of drug:protein (M_W = 230 (naproxen) and M_W = 14 400 (lysozyme)). Values are mean ± SD. Data are presented for three independent methods of drug analysis; i.e. by means of HPLC analysis of the drug (HPLC), fluorimetric drug analysis (F), and determination of unreacted amino groups by the method of Habeeb (H), as described in the Experimental Section. Degree of substitution as analyzed by the HPLC is also expressed as drug content of lyophilized conjugates (%, g/g); these data are subdivided in a covalently bound fraction and a noncovalently bound fraction (values in parentheses are presented as percentage of total amount of drug).

Table V. Identity and Purity of Drug–Spacer Derivatives as Obtained after Preparative HPLC

compd. no.[a]	parent peak[b]	theoretical MW	% purity[c]
1f	358 ($M^{\cdot+}$)	358	>99 (Ia)
1g	286 ($[M - H]^-$)	287	>99 (Ia)
1h	328 ($[M - H]^-$)	329	>99 (Ia)
1i	342 ($[M - H]^-$), 344 (MH^+), 361 ($M{\cdot}NH_4^+$)	343	>99 (Ia)
1j	376 ($[M - H]^-$)	377	>99 (Ia)
1k	392 ($[M - H]^-$)	393	>99 (Ia, II)
1l	415 ($[M - H]^-$)	416	>99 (Ia)
1m	358 ($[M - H]^-$)	359	>99 (Ia)
1n	385 ($[M - H]^-$)	386	>99 (Ia)
1o	456 ($[M - H]^-$), 458 (MH^+), 475 ($M{\cdot}NH_4^+$)	457	>99 (Ia)
1p	506 ($[M - H]^-$)	507	>99 (Ia)
1q	287 ($[M - H]^-$)	288	>99 (Ib)
1r	301 ($[M - H]^-$)	302	>99 (Ib)
2a	619/621 (MH^+), 636/638 ($M{\cdot}NH_4^+$)	618	>99 (III, IV)
2b	413/415 ($[M - H]^-$)	414	>99 (IV)
2c	469/471 ($[M - H]^-$)	470	>99 (III, IV)
2d	503/505 ($[M - H]^-$)	504	>99 (III, IV)
2e	519/521 ($[M - H]^-$)	520	>99 (IV)
2f	542/544 ($[M - H]^-$)	543	>99 (III, IV)
2g	485/487 ($[M - H]^-$)	486	>99 (III, IV)
2h	–	513	(III, IV)
3a	384 (MH^+), 401 ($M{\cdot}NH_4^+$)	383	>99 (VI)
3b	178 ($[M - H]^-$)	179	>99 (V, VI, VIIa)
3c	234 ($[M - H]^-$)	235	>99 (VI)
3d	268 ($[M - H]^-$)	269	>99 (VI, VIIa)
3e	284 ($[M - H]^-$)	285	>99 (VI, VIIa)
3f	307 ($[M - H]^-$)	308	>99 (II, VI)
3g	250 ($[M - H]^-$)	251	>99 (VI, VIIa)
3h	277 ($[M - H]^-$)	278	>99 (VI)
6a	466 ($[M - H]^-$)	467	>99 (Ia)
9a-I	367 (MH^+), 384 ($M{\cdot}NH_4^+$)	366	>99 (VIIb)
9a-II	367 (MH^+), 384 ($M{\cdot}NH_4^+$)	366	>99 (VIIb)
9a-III	367 (MH^+), 384 ($M{\cdot}NH_4^+$)	366	>99 (VIIb)
10a	408 ($[M - H]^-$)	409	>99 (X)

[a] Products are listed in Tables I and II. [b] Tabulated parent peaks are the observed molecular ions in the electron-impact or quasi-molecular ions observed in chemical ionization (CI-MS) mass spectra or ionspray mass spectra of the products. [c] Purity of the products is tabulated as established by HPLC. HPLC system numbers are in Roman numerals in parentheses and are described in the Experimental Section. Individual retention times are listed in Tables I and II.

the protein were determined by the method of Habeeb.[16] From these data the amount of reacted amino groups was calculated; the result, as is shown in Table IV, obviously corresponds with the amide formation with 1 and the molar degree of substitution. These three methods provide similar results and can be independently used for drug substitution measurements. Additional data with respect to the degree of substitution of conjugates with peptide spacers could be obtained by quantitative amino acid analysis (data not shown).

From SDS–PAGE and gel filtration experiments the absence of polymerized protein was ascertained (not shown).

The *N*-hydroxysuccinimide method was also used in the synthesis of many of the amino acid derivatives of the carboxylic model compounds **1g–p**, **2a–g**, **3a–h**, and **6a**). Selective protection of the α-amino group of lysine with a Z group was used in the preparation of ϵ-naproxen–lysine (**1f**). Deprotection was performed using catalytic reduction with Pd/H_2. Deprotection did not influence the nature of the parent drug. The ester derivatives (**1q–r**) were synthesized from the acid chloride derivative of **1**. The products were purified by preparative HPLC (Table V). Ester derivatives of **1** were also prepared on larger scale by condensation with DCC. The α-hydroxy acids were initially protected by a pentamethylbenzyl (PMB) group. After condensation and purification of unreacted reactants, this group was selectively removed by treatment with cold TFA in the presence of anisole. The anisole served to scavenge PMB cations which otherwise reacted with the aromatic nucleus of **1**. The naproxen–L-lactic acid derivative was further coupled to the lysines of lysozyme (**1s**) by a similar *N*-hydroxysuccinimide procedure. To examine the influence of spacer length, a tetra(L-lactic acid) spacer was intercalated by a similar procedure (**1t**).

Coupling of amino acids to the amino group model drugs was also performed by the *N*-hydroxysuccinimide method. Because of the reported leucine amino-peptidase activity in the lysosomes we decided to synthesize leucine–drug derivatives.[20] Protection of the amino acid (leucine) with a Boc group was necessary. The Boc group could be removed by the addition of TFA. After coupling and deprotection of Boc-Leu-NHS with triamterene, three different products resulted with an m/z ratio corresponding

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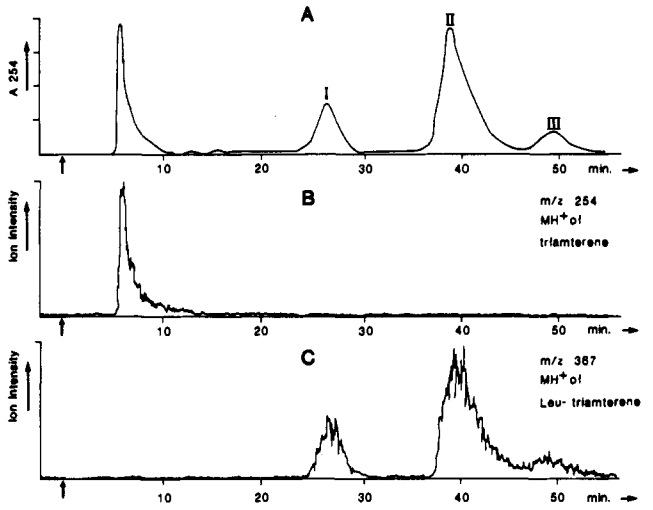

Figure 3. Example of product identification and purification by fractionated HPLC. The chromatographic pattern of the reaction mixture containing Leu-triamterene is shown (cp. **9a**-I–III). Three different products (cp. **9a**-I–III) were identified, separated, and isolated. Products are detected both by UV (254 nm) (panel A) and ion intensity (panels B and C).

to Leu-triamterene (**9a-I–III**, Figure 3). Reactions of the NHS esters of the protected amino acids with primary aromatic amino acid groups were either very slow (triamterene) or did not occur at all (sulfamethoxazole, not shown).

An acid-sensitive amide derivative of sulfamethoxazole was obtained by a reaction with *cis*-aconitic anhydride (**10a**). This derivative was further coupled to lysozyme (**10b**) through the γ-carboxyl group of the spacer molecule.

In Vitro Cleavage Studies in Renal Cortex Homogenates and Lysosomal Lysates. Cleavage data for the conjugates and derivatives of the model compounds and model drugs are listed in Tables I and II and are presented as percentage of released parent compound after 24 h of incubation. The catabolytes observed ultimately after incubation in lysosomal lysate are listed in the final column. Cleavage studies were carried out by incubating protein conjugates or drug derivatives with renal cortex homogenates or purified lysosomal lysates. Carboxypeptidase and aminopeptidase activities were estimated from the model compounds **4a**, **5a**, **7a**, and **7b**. Specific activities of the renal cortex homogenates were (nmol·s^{-1}·mg^{-1} protein): 0.47 (**5a**) and 0.092 (**7a**). Specific activities of the lysosomal lysates were (nmol·s^{-1}·mg^{-1} protein) 0.80 (**5a**) and 0.062 (**7a**). The lysosomal lysates showed a 5–6 times enrichment of lysosomal enzymes as determined by the lysosomal enzyme marker acid phosphatase. Incubations with the renal homogenates were performed at pH 5.0 and 7.4 to mimic lysosomal and cytosolic digestion, respectively.

The cleavage data for the terminal carboxyl group containing model compounds after 24 h are shown in Table I. Although incubation of the 1-lysozyme conjugates (**1a–e**) with the homogenates did not reveal any detectable 1, catabolytes with a single amino acid were observed, e.g. Nap–Lys and Nap–Leu. In this respect all 1-amino acid spacers always resulted in catabolytes with the one single terminal amino acid as ultimate catabolyte (**1f–p**). In addition, incubation did not lead to any other detectable metabolites of 1, such as 6-desmethylnaproxen in either conjugated (as sulfate or glucuronide) or unconjugated form. Similar observations were made when indomethacin and benzoic acid were used as model drugs (cp. **2a–h** and **3a–h**).

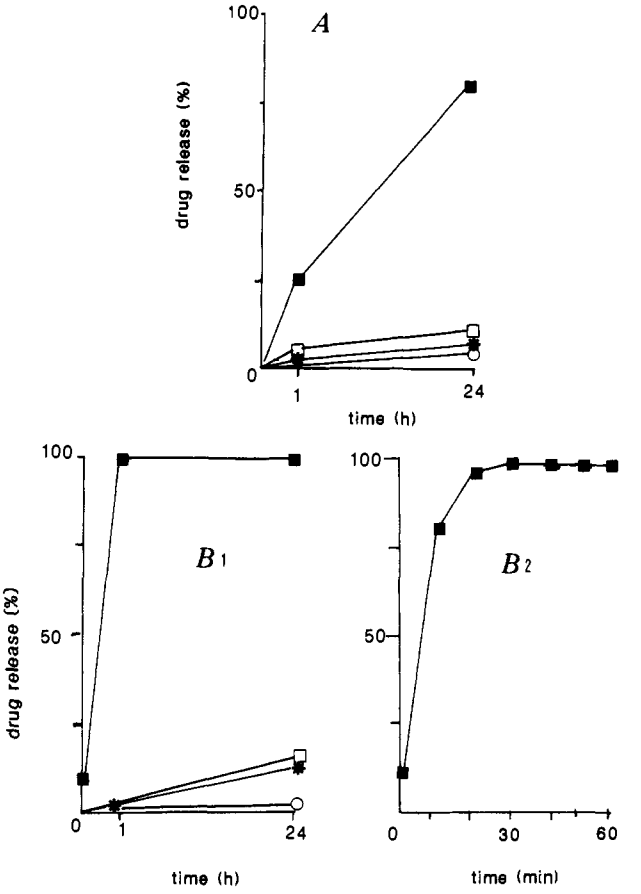

Figure 4. Drug release versus time profile after incubating naproxen–glycolic acid (10 μM; A) and naproxen–L-lactic acid (10 μM; B1 and B2) with lysosomal lysate, pH 5.0, ■; lysosomal lysate, pH 7.4, □; plasma, pH 7.4, *; blank, pH 5.0, ○.

On the other hand the model compounds Z-Phe-Ala (cp. **4a**), Z-Pro-Phe (cp. **5a**), and Z-Ala-Pro-Phe (cp. **6a**) were rapidly degraded to their parent forms (within 1 h).

The ester derivatives of naproxen (cp. **1q** and **1r**) were rapidly cleaved in homogenate and lysosomal lysate at pH 5.0. Figure 4 shows the drug release versus time profiles of equimolar amounts (10 μM) of the glycolic and L-lactic derivatives in the cortex homogenates at pH 5.0. An 81% release of the naproxen from the glycolic spacer occurs within 24 h, whereas, within 30 min, 100% of the naproxen is released from the L-lactic acid spacer. In plasma and also in the homogenate and lysate at pH 7.4., these compounds were rather stable: 8% and 13% release, respectively, within 24 h. Further coupling to lysozyme of **1r** resulted in a decreased rate of naproxen regeneration. Increasing the spacer length by intercalating additional L-lactic acid subunits resulted in a 6-fold increase in drug regeneration.

The cleavage data for the terminal amino group containing model drugs are shown in Table II. The leucine amino acid derivatives of the amino model compounds β-naphthylamine (**7a**) and adriamycine (8) were cleaved to their parent form. However, Leu-triamterene was found to be stable after 24 h of incubation.

The *cis*-aconitic amide derivative of sulfamethoxazole (**10a**) was found to be cleavable after incubation and resulted in the parent sulfamethoxazole. Its corresponding lysozyme derivative was also found to be cleavable (**10b**).

Discussion

The *N*-hydroxysuccinimide procedure appears to be a convenient method for drug–protein conjugation and the

synthesis of many drug–oligopeptide derivatives. Furthermore, since the drug or its spacer derivative is converted to an NHS ester derivative in the absence of protein, intra- or intermolecular cross-linking does not occur. Estimation of drug content after coupling to the protein can be done by HPLC after its acid or alkaline hydrolysis from the conjugates. This method also allows discrimination between covalently and noncovalently bound drug.

Renal cortex homogenates and lysosomal lysates were used to study degradation of conjugates and drug–spacer derivatives. This method allowed a rapid screening of catabolytes of different compounds and may be used as an initial probe prior to determination of in vivo pharmacokinetics of protein–drug conjugates.

Both preparations showed pronounced carboxypeptidase and aminopeptidase activity. However, in vitro cleavage studies per se may be limited by factors related to purification artefacts. These factors are, for instance, inactivation of enzymes by the isolation procedure, or lack of essential cosubstrates. In this study we tried to limit these shortcomings by incubating with both complete cortex homogenates as well as purified lysosomal lysates.

A direct linkage of the carboxylic acid model drug 1 to lysozyme resulted in the generation of ϵ-1–lysine as ultimate catabolyte (**1a**). Obviously such ϵ-amide bonds are not well-recognized by or are poorly accessable for the hydrolases. Intercalation of oligopeptide spacers was expected to result in physiologically more readily cleavable α-amide bonds and improvement of the accessability toward lysosomal enzymes.[17] However, also intercalation of some oligopeptide spacers resulted in catabolytes with a single amino acid bound to the drug (**1b–e**). Again neither free 1 nor other metabolites were observed. Obviously, the choice of a suitable type of spacer (i.e. type of amino acids and spacer length) is very critical and is dependent on the availability of selective proteolytic enzymes in the lysosomes. Therefore we decided to study the cleavability of individual model drug–amino acid compounds. These derivatives may be regarded as catabolytes, which are formed during proteolysis in lysosomes and ultimately have to release parent active drug for successful drug targeting. The amino acids tested varied in their properties with respect to hydrophobicity (Leu > Val > Gly), presence of aromatic side chains (Trp, Tyr, Phe), and charge (Glu, Arg). The ϵ-drug–lysine derivatives may be regarded as models for COOH-containing compounds directly linked to amino group side chains of proteins. However, amino acid and tripeptide derivatives of naproxen did not result in the generation of 1 or its main metabolite 6-desmethylnaproxen, but rather in the formation of 1 attached to a single amino acid (**1g–p**). Moreover, all the α-amide linkages of the carboxyl group containing model compounds 1–3 with the selected natural amino acids appeared to be resistant to enzymatic hydrolysis. Only in the exceptional case that the model drug itself contained a natural amino acid, as is shown by the testing of model compounds Z-Phe-Ala (**4a**), Z-pro-Phe (**5a**), and Z-Ala-Pro-Phe (**6a**), was the parent compound generated.

Obviously the carboxypeptidases in the kidney are quite specific and this will limit formation of the active parent drug in the case of a direct linkage of the drug to the protein but also after linkage of the drug to a peptide spacer. Metabolism of such conjugates may lead to oligopeptide catabolytes, which will not further degrade to their active parent forms. However, in particular cases, as has been reported for some cytostatic drugs, these fragments may still have some of the activity of the parent form and therefore such conjugates may still be of use in selective drug delivery.[18–20] In support of this idea, we found a clear antiinflammatory activity of the ultimate catabolyte of the 1–lysozyme conjugate ϵ-naproxen–lysine.[21]

Therefore we conclude that for nonsteroidal antiinflammatory drugs (NSAID's) and other structurally related agents, a peptide spacer concept is no guarantee for rapid and efficient liberation of the parent drug. Moreover, on pharmacokinetic grounds, rapid digestion seems strongly required for these kinds of drugs. These carboxyl group containing drugs are weak acids and are therefore in an uncharged lipophilic form at low pH values. Also the generated oligopeptide catabolytes are weak acids, and since the pH's in the lysosomal and cytosolic compartment are reported to be 4–5 and 7.4, respectively, these catabolytes may rapidly leave the lysosomal compartment to the cytosolic compartment in their nonionized form by passive diffusion and thereby possibly escape lysosomal digestion. Our observations concerning release of amino acid fragments of NSAID's in the kidney are in agreement with the observations of Renard et al.[22] concerning the catabolism of a model compound for penicillin V in the liver. In this study Boc-D-Pro-Ala was studied as model compound for penicillins in liver lysosomal extracts and in contrast to Boc-L-Pro-Ala incubations, which result in a 70% release of free Ala, only 7% of free Ala was released after a 24-h incubation. Furthermore these authors also examined some derivatives of penicillins with amino acids, which were also found to be resistant to enzymatic hydrolysis.

Only in exceptional cases of peptide type drugs, as is shown by model compounds **4a–6a**, may an amide linkage cleave to provide the parent drug. On the other hand, the drug itself has to be stable in the lysosomal compartment. In this respect, our model compound **6a** seems very interesting. Apparently, this small peptide is selectively cleaved by kidney prolinases between proline and phenylalanine; the ultimate catabolyte is Z-Ala-Pro, which itself is not further degraded by the proteolytic enzymes. Since the parent compound **6**, Z-Ala-Pro, shows remarkable structural similarity with the potent angiotensin-converting-enzyme inhibitor enalaprilate, the peptide spacer concept seems promising for these kinds of drugs (Chart III).

Since for many of the carboxyl group containing drugs obviously an amide bond will not lead to generation of the parent drug, we examined the use of an ester bond. Glycolic acid and L-lactic acid derivatives of 1 were rapidly cleaved by esterases in the lysosomal lysates. Further coupling of the L-lactic acid derivative **1r** by its free carboxyl group to the lysines of lysozyme gave rise to conjugate **1s**, obviously more resistant to enzymatic attack in the lysosomes. We hypothesized that the ester bond is less accessable for the esterases. Chain lengthening by intercalating a tetra(L-lactic acid) spacer indeed made the bond more susceptible for enzymatic attack (**1t**). This observation that spacer length is critical for enzymatic attack of ester bonds in renal lysosomes is analogous to those reported by Trouet et al. for amide bonds in liver lysosomes.[17] Further, these ester LMWP conjugates are sufficiently stable in plasma. LMWPs are rapidly cleared

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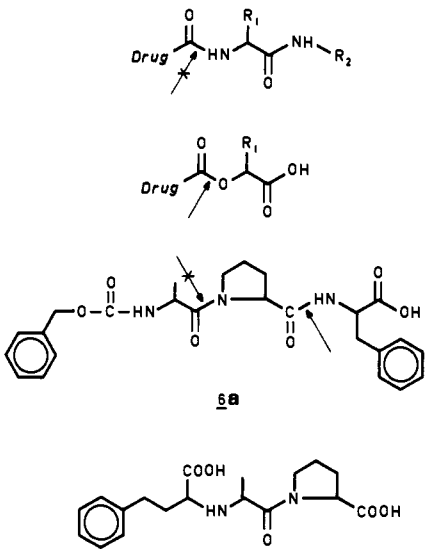
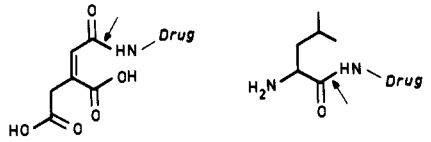
Chart III[a]



6a

[a] Amide derivatives of model compounds and model drugs with a carboxyl group are not cleaved by carboxypeptidases present in the renal cortex homogenates and lysosomal lysates. Only such derivatives of amino acid containing models (like derivative 6a of model compound 6) are cleaved by pepidases or amidases (bottom structure represents enalaprilate). Ester derivatives of these compounds are cleaved by carboxyesterases.

Chart IV[a]



[a] Amide derivatives of model compounds and model drugs with a primary amino group are cleaved by aminopeptidases present in the renal cortex homogenates and lysosomal lysates or the drug is released from an acid sensitive *cis*-aconityl spacer.

from the bloodstream by glomerular filtration. In case of an intravenous injection of 10 mg native lysozyme in rats, the plasma clearance is 1.2 mL·min^{-1}. Roughly 50% of this dose is reabsorbed in the kidneys and locally metabolized; 50% of the dose is excreted as unchanged protein in the urine (recovered within 2 h after injection). Therefore, exposure to plasma esterases during transport to the kidney will be very short. In this respect, plasma stability of various ester derivatives of benzoic acid as model compounds were examined by Bundgaard.[23] These were also found to be stable. In conclusion, these drug–proteins, equipped with ester spacers, release parent drug in contrast to their amide spacer equipped analogues. Thus these conjugates are suitable candidates for the renal drug targeting issue.

In contrast to the carboxyl group containing model drugs, the amino group containing model drug derivatives Leu–β-naphthylamine and Leu–adriamycine release their parent drug during lysosomal incubation. Apparently, these compounds can be cleaved by the aminopeptidases in the kidney. However, leucine derivatives of triamterene (**9a-I**, **-II**, and **-III**) were not cleaved by the lysosomal enzymes in the cortex of the kidney.

We conclude that for the model drugs the aminopeptidases in the kidney exhibit a broader specificity than the carboxypeptidases. Therefore, a peptide spacer concept seems promising for various amino group terminal drugs. This conclusion is further supported by the fact that several oligopeptide spacers indeed have been described for some compounds with a terminal amino group. For example, for the cytostatic drug daunorubicin (DNR) the tetrapeptide spacer Ala-Leu-Ala-Leu-DNR has been described. Incubation with liver lysosomal lysates resulted primarily in several oligopeptide catabolytes, which were ultimately cleaved by aminopeptidases to provide parent daunorubicin.[17] Moreover, catabolytes like Leu–daunorubicin and Leu–adriamycin will enzymatically degrade to their parent form, as has been shown in liver lysosomal preparations[20] and in suspensions of rabbit renal proximal tubules.[24] Note that a biodegradable pentapeptide spacer Gly-Phe-Leu-Gly-Phe has been described for the model compound *p*-nitroaniline.[13,25,26]

However, extrapolation of cleavage studies of oligopeptide spacer–model compounds to other drugs may be limited by the nature of the drug itself. In our study this is exemplified by the observed lack of generation of free triamterene from its leucine derivatives, in spite of the fact that these derivatives show some structural similarity with leucine–β-naphthylamine (i.e. also a primary aromatic amine function, Chart II). Only the latter model compound is converted to parent naphthylamine. Furthermore, it has been reported by Lloyd et al.[27] that a polymer-bound oligopeptide that readily released the drug analogue *p*-nitroaniline failed to release another structurally related but unspecified drug.

The peptide spacer concept for these drugs is also interesting from the pharmacokinetic viewpoint. In contrast to their acid carboxyl group containing counterparts, the amino terminal fragments generated may become entrapped in the lysosomal compartments, since they are predominantly in their ionized form at pH 5, restricting passage of the lysosomal membrane. This means in principle a prolonged exposure to lysosomal digestion and therefore a higher chance of parent drug generation.

To overcome problems related to enzyme specificity, an acid-labile spacer may be of use. Use of available spacers have been limited to that as coupling agent for the amino group compounds. In this concept, the drug is expected to be released from the spacer in the acid environment of the endosomes and lysosomes; enzymatic activity is not required for drug release. An acid-labile amide bond for the cytostatic drug daunorubicin has been described by Shen et al.[28] In the present study, this concept is supported by the rapid release of sulfamethoxazole from lysozyme using the same acid-sensitive linker (**10b**). Therefore the option of a hydrolytically labile bond is attractive for these kinds of drugs.

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(24) Hjelle, J. T.; Baurain, R.; Masquelier, M.; Trouet, A. Cellular Pharmacology of Amino Acid Derivatives of Daunorubicin and Doxorubicin in Suspension of Renal Proximal Tubules. *J. Pharmacol. Exp. Ther.* **1984**, *229*, 372.

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(27) Lloyd, J. B.; Duncan, R.; Kopecek, J. Synthetic Polymers as Targetable Carriers for Drugs. *Pure Appl.* Chem. **1984**, *56*, 1301.

(28) Shen, W. C.; Ryser, J. P. Cis-aconityl Spacer between Daunomycin and Macromolecular Carriers: a Model of pH-sensitive Linkage Releasing Drug from a Lysosomotropic Conjugate. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 1048.

General Conclusions

Characterization of drug–LMWP conjugates requires proper analysis of both protein and drug content. Drug content estimation after its coupling to a LMWP can be performed by HPLC methods after alkaline or acid hydrolysis of the conjugates. In addition these methods can also discriminate between covalently and noncovalently bound drug.

For the synthesis of suitable drug–LMWP conjugates it is essential that the bond between the drug and the protein is degradable in the renal lysosomes. Otherwise no parent drug will be regenerated. We have shown that the option of enzymatic cleavage of such bonds is limited by the narrow specificity of the renal lysosomal enzymes. This clearly restricts the number of drugs and types of bonds. Tailor-made spacers are required for this option of drug release. The option of chemical hydrolysis using acid-sensitive linkers is therefore an attractive alternative approach.

For drugs with carboxyl groups, spacers with ester bonds are required for drug release. Esterases are capable of cleaving such bonds. Increasing spacer length makes the bond more accessable for the enzymes. In general for these kinds of drugs amide bonds are not cleaved by renal carboxypeptidases. Amide bonds are only suitable for use with drugs with peptide structures themselves. Whether aminopeptidases will be capable of cleaving amide bonds of amino group containing drugs will be dependent on the nature of the drug itself. For drugs with terminal amino groups, an acid-sensitive amide linkage is an attractive alternative.

Experimental Section

Chemicals and Reagents. All chemicals and reagents were of synthetic or analytical grade, as were all chemicals for HPLC purification and analysis. Water for HPLC analysis was of Millipore quality. Naproxen, lysozyme (3X crystallized, grade I), (protected) amino acids and oligopeptides, Leu-naphthylamide, and Gly-Phe-naphthylamide were purchased from Sigma. Leu-adriamycin and Gly-Leu-adriamycin were donated by C. Hoes (Organon B.V., Oss, The Netherlands). Tetra(L-lactic acid) (Mu (estimated mean molecular weight) = 315; Pu (estimated average number of contituents) = 4.2) was supplied by D. Grijpma (Department of Polymer Chemistry, University of Groningen).

Synthesis. General. The following procedures will be used for description of compounds and procedures. The synthetic procedures for the preparation of the various naproxen intermediates are described below. The identity and purity of the compounds were checked by ^{1}H NMR, mass spectrometry, and elemental analysis. The identity and purity of native proteins and their drug conjugates were established by SDS-PAGE and FPLC chromatography (Pharmacia). Relevant drug substitution data are tabulated in Tables III and IV. Drug–spacer derivatives were prepared on either a large or a small scale. Large-scale syntheses are described below. Products of the small-scale synthesis (<50 μmol of model compound or model drug) were obtained by fractionated HPLC, after evaluating the chromatographic patterns of the reaction mixtures together with those of corresponding blank syntheses (i.e. without the presence of the spacer in the reaction vial). General procedures: S1–S7 refer to Tables I and II. Each general procedure was used for the preparation of those derivatives listed under this heading in Tables I and II. Those compounds not bearing an S number in Tables I and II were either purchased or the synthesis is described below. Each such derivative was identified by mass spectrometry of the pure compound or combined HPLC/mass spectrometry (LC–MS). In all cases the observed parent peak corresponded to the theoretical molecular mass of the derivative. Identity and purity of the miniscale products are tabulated in Table V.

Melting points were measured on a Heidolph-Schwabach 30 melting point apparatus (Hilversum). Infrared spectra were recorded on a Jasco A-100 and UV spectra on a PU 8700 UV/VIS scanning spectrophotometer (Philips). Fluorescence spectra were measured on a SLM-Aminco 500C apparatus. ^{1}H NMR spectra were taken at 60 MHz on a Perkin-Elmer or a JEOL C-60 HL and at 300 MHz on a Varian VXR 300. EI and CI mass spectra were obtained on a Finnigan 3300 instrument. A Nermag R 3010 triple quadrupole mass spectrometer equipped with an ion spray interface[29] was used for LC–MS. Chromatographic separations by means of high-performance liquid chromatography were performed on a HPLC system with a μ-Bondapack C-18 column (Waters/Millipore Corp., Milford, MA). The system was equipped with an autoinjector (WISP), UV detector (Waters 440), and a fluorescence detector (Waters 470). The various eluent conditions for the different derivatives are described in the chromatography section. Individual retention times are listed in Tables I and II.

6-Desmethylnaproxen (6-DMN). Naproxen was dried (vacuum, 70 °C) and 230 mg (1 mmol) was suspended in 10 mL of a 47% HBr solution. The suspension was refluxed for 1 h under a nitrogen atmosphere whereupon a clear solution was obtained. Upon cooling on ice a precipitate was formed; this was filtered, washed with ice/water, and crystallized from MeOH/EtOH/water. ^{1}H NMR, IR, and mass spectrometry for 6-desmethylnaproxen were in agreement with the literature.[30]

Naproxen *N*-Hydroxysuccinimide Ester (Nap-NHS). Naproxen (1.725 g, 7.5 mmol) was dissolved in 50 mL of dichloromethane and DCC (2.063 g, 10 mmol) was added. The solution was stirred for 15 min. Thereafter NHS (1.150 g, 10 mmol), dried at 50 °C in vacuo for 24 h, was added; the mixture was stirred for 24 h. After filtration of the precipitate the filtrate was evaporated in vacuo and the residue was washed with dry heptane. The residue was dissolved in ethyl acetate, filtered, evaporated in vacuo, and crystallized from dichloromethane/hexane to afford Nap-NHS in 91% yield (n = 4). Identity and purity were verified by ^{1}H NMR, ^{13}C NMR, MS, HPLC, and elemental analysis. ^{1}H NMR ($CDCl_3$): δ 7.8–7.1 (m, 6, aromatic *H*), 4.19 (q, 1, C*H*Me), 3.91 (s, 3, CH_3O), 2.80 (s, 4, $-CH_2CH_2-$, (NHS)), 1.71 (d, 3, CH_3C). ^{13}C NMR ($CDCl_3$): δ 18.7 (CH_3), 25.3 (CH_2), 42.7 (CH), 55.1 (CH_3), 105.4 (CH), 119.0 (CH), 125.7 (CH), 126.1 (CH), 127.3 (CH), 128.6 (Cq), 129.1 (CH), 133.1 (Cq), 133.7 (Cq), 157.6 (Cq), 168.8 (Cq), 169.8 (Cq). IR (KBr): 1715, 1775, 1810, absence of broad peak at 3400–3050 cm^{-1} (OH). CI-MS: m/z 328 (MH^+), 345 ($M + NH_4^+$), theoretical MW = 327. Purity > 99.5% as established by HPLC (system Ia): t_R = 24.0 min. Mp: 112–114 °C. Anal. ($C_{18}H_{17}NO_5$): C, H, N.

Naproxen Acid Chloride (Nap-Cl). Naproxen (920 mg, 4 mmol) was dissolved in 50 mL of toluene to which 5 mL of $SOCl_2$ was added. Two drops of DMF were added and the suspension was refluxed for 3 h. The mixture was evaporated in vacuo, stripped with toluene, and crystallized from dichloromethane/hexane. The yield was 95% and the identity and purity were verified by MS, ^{1}H NMR, TLC, IR, and elemental analysis. ^{1}H NMR ($CDCl_3$): δ 7.8–7.1 (m, 6, aromatic), 4.1 (q, 1, C*H*Me), 3.9 (s, 3, CH_3), 1.5 (d, 3, CH_3C). IR (NaCl): 1785, 1600 cm^{-1}. Anal. ($C_{14}H_{13}O_2Cl$): C, H, Cl.

Naproxen–Leucine–Lysozyme (cp. 1b). (a) Preparation of the Ethyl Ester of Leucine. Leucine (1.3 g, 10 mmol) was suspended in 50 mL of absolute ethanol and the mixture was stirred at −5 °C. Two drops of DMF were added. Thereafter 1.5 mL of thionyl chloride (1.6 g/mL, 20 mmol) was added, and the temperature was kept below 0 °C. The temperature was elevated to ambient. Thereafter the mixture was refluxed for 2 h. The solvent was evaporated in vacuo. Water was added and the pH was adjusted to 7–8 with $NaHCO_3$. The solution was extracted with 3 × 20 mL of dichloromethane. The organic layers were combined and washed with 2 × 15 mL of 0.5 M $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, filtered off, and evaporated in vacuo.

(b) Synthesis of *N,N'*-Thionyldiimidazole. Imidazole (1.83 g, 25 mmol), previously dried over phosphorus pentoxide, was dissolved in 100 mL of dry THF; 0.44 mL of thionyl chloride (714 mg, 6 mmol) was added under a nitrogen atmosphere. The so-

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lution was stirred for 1.5 h. The precipitate formed was filtered off. The filtrate was kept at 0 °C and immediately used for reaction c.

(c) Synthesis of Naproxen-Leucine Ethyl Ester. Naproxen (1.38 g, 6 mmol) was dissolved in 10 mL of dry THF and added to the cooled freshly prepared *N,N'*-thionyldiimidazole solution. The solution was placed in an ice/salt bath (-5 °C) and was stirred for 0.5 h. Leucine ethyl ester (2.23 g, 6 mmol) was suspended in 10 mL of dry THF. The suspension was cooled on ice and thereafter slowly added to the naproxen-*N,N'*-thionyldiimidazole solution. The mixture was stirred for 0.5 h. TEA (606 mg, 6 mmol), previously dissolved in a small portion of dry THF, was added. The reaction mixture was raised to ambient temperature and the mixture was stirred for 24 h. The mixture was filtered off and the filtrate was evaporated in vacuo to a final volume of 5 mL. The filtrate was acidified with 1 N HCl and diluted with a small portion of water (pH solution = 3). The product was extracted with 3 × 50 mL of dichloromethane. The organic layers were combined and washed with four portions of $NaHCO_3$ buffer (1 M, pH 9) to remove unreacted naproxen. The absence of unreacted naproxen (<0.1%) was verified by HPLC (system Ia). The organic layer was dried over anhydrous sodium sulfate, filtered off, and evaporated in vacuo. The product was crystallized from dichloromethane/hexane. The yield was 85%. ^{1}H NMR ($CDCl_3$): δ 7.40 (m, 6, aromatic *H*), 5.75 (d, 1, -N*H*-), 4.75 (m, 1, NC*H*CO (Leu)), 4.10 (q, 2, OCH_2C (Et)), 3.91 (s, 3, CH_3O), 3.74 (q, 1, C*H*Me (naproxen)), 1.61 (d, 3, CCCH_3 (naproxen), 1.55 (m, 2, CCH_2C (Leu)), 1.40 (t, 1 Me_2C*H*C (Leu)), 1.18 (t, 3, CH_3 (Et)), 0.88 (d, 3, CH_3 (Leu)), 0.86 (d, 3, CH_3 (Leu)). CI-MS *m/z* 372 (MH^+). Anal. ($C_{22}H_{29}NO_4$): C, H, N.

(d) Synthesis of Naproxen-Leucine (OH) (cp. 1i). Nap-Leu-OEt (1.11 g, 3 mmol) was dissolved in 20 mL of MeOH/water (50/50 (v/v)). A 1 M LiOH solution (6 mL, 144 mg, 6 mmol) was added and the reaction was followed by TLC. After completion of hydrolysis, the solution was acidified to pH 2.5–3. MeOH was evaporated in vacuo and the water layer was extracted with 3 × 25 mL of diethyl ether. The organic layers were combined and dried over anhydrous Na_2SO_4, filtered off, and evaporated in vacuo. The product was crystallized from dichloromethane/hexane. The yield was 95%. ^{1}H NMR (CD_3OD): δ 8.60 (s, 1, O*H*), 7.8–7.1 (m, 6, aromatic), 5.91 (d, 1, -N*H*-), 4.62 (q, 1, NC*H*CO (Leu)), 3.86 (s, 3 CH_3O), 3.74 (q, 1, C*H*Me (naproxen), 1.55 (d, 3, CH_3), 1.45 (m, 3, CCH_2C*H*Me_2 (Leu)), 0.85 (dd, 6, (CH_3)$_2$C (Leu). ^{13}C NMR (CD_3OD): δ 18.8 (CH_3), 21.9 (CH_3), 26.0 (CH), 41.6 (CH_2), 46.9 (CH), 52.0 (CH), 55.6 (CH_3), 106.4 (CH), 119.7 (CH), 126.7 (CH), 127.3 (CH), 127.9 (CH), 130.1 (CH), 130.2 (Cq), 135.0 (Cq), 137.5 (Cq), 158.8 (Cq), 175.8 (Cq), 177.1 (Cq). IR (KBr): 1680, 1654, 1600 cm^{-1} (CONH). Mp: 127–130 °C. EI-MS: *m/z* 343 (M^+), 325 [(M - OH)$^+$]. Anal. ($C_{20}H_{25}NO_4$): C, H, N.

(e) Synthesis of Naproxen-Leucine NHS Ester. Naproxen-Leu(OH) (343 mg, 1 mmol) was dissolved in 10 mL of chloroform. Thereafter DCC (277 mg, 1.1 mmol) was added. The solution was stirred for 15 min. NHS (115 mg, 1 mmol), previously dried at 50 °C in vacuo for 24 h, was added; the mixture was stirred for 24 h. The mixture was further treated as described for Nap-NHS. The yield was 94%. ^{1}H NMR ($CDCl_3$): 7.8–7.1 (m, 6, aromatic *H*), 5.9 (d, 1, N*H*), 4.6 (q, 1, NC*H*CO (Leu)), 3.9 (s, 3, CH_3O), 3.7 (q, 1, C*H*Me (naproxen)), 2.8 (s, 4, CH_2CH_2 (NHS)), 1.6 (d, 3, CH_3), 1.5 (m, 3, CCH_2CHMe_2 (Leu)), 0.9 (m, 6, (C*H*)$_3$C (Leu)).

(f) Synthesis of Naproxen-Leucine-Lysozyme (cp. 1b). Nap-Leu-NHS (22 mg, 0.05 mmol) was dissolved in 2.2 mL of 1,4-dioxane and allowed to react with lysozyme (766 mg, 0.05 mmol) as described under S7. The molar degree of substitution was 0.3, as established by fluorimetric naproxen assay and Bradford protein assay.[32]

Naproxen-Leucine-Glycine-Glycine-Lysozyme (cp. 1c).

(a) Synthesis of Leu-Gly-Gly-OEt. A similar procedure was used as described for Leu-OEt.

(b) Synthesis of Naproxen-Leu-Gly-Gly-OEt. Naproxen (1.40 g, 6.1 mmol) was allowed to react with Leu-Gly-Gly-OEt (1.67 g, 6.1 mmol) as described for naproxen-Leu-OEt. After recrystallization from dichloromethane/hexane pure product was obtained. The yield was 82%. IR (KBr): 3295, 1740, 1655 cm^{-1}. CI-MS: *m/z* 486 (MH^+), 504 (M + NH_4^+).

(c) Synthesis of Nap-Leu-Gly-Gly(OH) (cp. 1o). Nap-Leu-Gly-Gly-OEt (1.46 g, 3 mmol) was hydrolyzed to yield Nap-Leu-Gly-Gly(OH) exactly as described for Nap-Leu(OH), using dichloromethane instead of diethyl ether as extraction solvent. A pure product was obtained. The yield was 90%. Mp: 79–82 °C. ^{1}H NMR (CD_3OD): δ 8.3–7.1 (m, 10, aromatic *H*, N*H* (3), O*H*), 4.4 (q, 1, NHC*H*C (Leu)), 3.9 (s, 3, CH_3O (naproxen), 3.8 (s, 4, CH_2 (2) (Gly)), 3.7 (q, 1, C*H*CH_3 (naproxen)), 1.6 (d, 3, CH_3 (naproxen)), 1.5 (m, 3, CCH_2C*H*(CH_3)$_2$ (Leu)), 1.0 (dd, 6, (CH_3)$_2$C (Leu)). ^{13}C NMR (CD_3OD): δ 16.8 (CH_3), 20.1 (CH_3), 21.3 (CH_3), 23.8 (CH), 39.1 (CH_2), 39.6 (CH_2), 41.3 (CH_2), 44.8 (CH), 51.6 (CH), 53.7 (CH_3), 104.4 (CH), 117.8 (CH), 124.7 (CH), 125.I (CH), 126.0 (CH), 128.1 (CH), I32.9 (Cq), 135.3 (Cq), I56.8 (Cq), 169.4 (Cq), 170.7 (Cq), 173.0 (Cq), 175.6 (Cq). CI-MS: *m/z* 458 (MH^+). Anal. ($C_{24}H_{31}N_3O_6$): C, H, N.

(d) Synthesis of Nap-Leu-Gly-Gly NHS Ester. Nap-Leu-Gly-Gly(OH) (457 mg, 1 mmol) was dissolved in 10 mL of chloroform. Thereafter DCC (227 mg, 1.1 mmol) was added. The solution was stirred for 15 min. NHS (115 mg, 1 mmol), previously dried at 50 °C in vacuo for 24 h, was added; the mixture was stirred for 24 h. The mixture was further treated as described for Nap-NHS. The yield was 98%. ^{1}H NMR: 8.3–7.1 (m, 9, aromatic *H* (6), N*H* (3)), 4.4 (q, 1, NHC*H*C (Leu)), 3.9 (s, 3, CH_3O (naproxen)), 3.8 (s, 4, CH_2 (2) (Gly)), 3.7 (q, 1, C*H*CH_3 (naproxen)), 2.8 (s, 4, -CH_2CH_2- (NHS)), 1.6 (d, 3, CH_3 (naproxen), 1.5 (m, 3, CCH_2C*H*(CH_3)$_2$ (Leu)), 1.0 (dd, 6, (CH_3)$_2$C (Leu)).

(e) Synthesis of Naproxen-Leucine-Glycine-Glycine-Lysozyme (cp. 1c). Nap-Leu-Gly-Gly-NHS ester (28 mg, 0.05 mmol) was dissolved in 2.8 mL of 1,4-dioxane and allowed to react with lysozyme (766 mg, 0.05 mmol) as described under S7. The molar degree of substitution was 0.4, as established by fluorimetric drug assay and protein assay by the method of Bradford.[32]

ε-Naproxen-Lysine (ε-Nap-Lys, cp. 1f). The synthesis of ε-Nap-Lys-OEt was carried out as described by Grolleman et al.[31] Under a dry nitrogen atmosphere naproxen (1.83 g, 8.0 mmol) and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) (1.97 g, 8.0 mmol), freshly dissolved in 65 mL of dry dichloromethane, were stirred at room temperature. After 1 h α-Z-lysine ethyl ester (2.7 g, 8.8 mmol) in 16 mL of dichloromethane was added. The solution was stirred for 2 h. Thereafter the solvent was evaporated. White crystals were formed during storage overnight at 5 °C. After filtration, washing with diethyl ether, and drying, 2.67 g (65%) of the desired product was obtained (mp: 119 °C). Deprotection of the Z group was carried out by addition of 100 mg of palladium on active carbon (Pd/carbon) to a solution of the product (2.67 g, 0.05 mmol) in ethanol in a dry nitrogen atmosphere. The mixture was allowed to react with hydrogen under atmospheric pressure and the liberated carbon dioxide was absorbed by solid NaOH. After 18 h the reaction was complete (checked by the disappearance of the benzyl group by ^{1}H NMR). Pd/C was filtered off and after evaporation a clear oil was obtained which was dissolved in chloroform and washed with two portions (15 mL each) of saturated $NaHCO_3$ solution and subsequently with a small portion of water. The chloroform layer was dried over anhydrous Na_2SO_4, filtered, evaporated in vacuo, and dried in vacuo for 24 h until crystallization occurred (1.18 g, 60%). The ^{1}H NMR spectra and mass spectra were in agreement with those reported in the literature.[31] Pure ε-naproxen-lysine(OH) (ε-Nap-Lys) was obtained by the addition of 2 drops of 1 N NaOH to a methanolic solution of ε-Nap-Lys-OEt; after 30 min the reaction was complete as checked by HPLC (system Ia; t_R: ε-

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Nap-Lys-OEt, 7.3 min; ϵ-Nap-Lys(OH), 3.3 min). EI-MS m/z 358 (M^+).

Naproxen-L-Lactic Acid-Lysozyme. (a) Synthesis of L-Lactic Acid PMB Ester. The L-lactic acid PMB ester was synthesized by the method of Steward et al.[36] A suspension of L-lactic acid (1.25 g, 10 mmol) in DMF was treated with TEA (20 mmol) and pentamethylbenzyl chloride (PMBCl) (10 mmol). PMBCl was synthesized by the method of Aitken, et al.[37] The mixture was warmed gently to effect solution and kept overnight at room temperature. Thereafter, an excess of 1 N NaHCO, was added. The ester separated in crystalline form within a few minutes. The product was collected, washed with water, and dried under vacuum. The yield was 95%. Mp: 115–116 °C. ^{1}H NMR ($CDCl_3$): δ 5.27 (m, 2, CH_2), 4.23 (q, 1, $CHCH_3$), 2.27 (s, 15, CH_3-Cq), 1.47 (d, 3, CH_3CH).

(b) Synthesis of Naproxen-L-Lactic Acid PMB Ester. Condensation of naproxen with L-lactic acid PMB ester was done by the procedure of Gilon et al.[38] for the synthesis of depsipeptides. To a solution of naproxen (2.3 g, 10 mmol), L-lactic acid PMB ester (2.5 g, 10 mmol), and 4-DMAP (1.22 g, 10 mmol) in 150 mL of dichloromethane was added a solution of DCC (2.27 g, 11 mmol) in 50 mL of dichloromethane. The reaction mixture was stirred at 25 °C and followed by TLC. The *N,N*-dicyclohexylurea was filtered off. The filtrate was washed with 1 M $KHSO_4$ (2 × 20 mL), water (2 × 20 mL), and 5% $NaHCO_3$ (2 × 20 mL); the organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. The residue was washed with petroleum ether and left several hours under high vacuum to afford analytically pure product. The yield was 70%. ^{1}H NMR ($CDCl_3$): δ 7.69–7.12 (m, 6, aromatic), 5.27 (m, 2, CH_2), 5.10 (q, 1, $CHCH_3$ (lact)), 3.94 (q, 1, $CHCH_3$ (naproxen)), 3.93 (s, 3, CH_3O), 2.27 (s, 15, CH_3-Cq), 1.61 (d, 3, CH_3CH (naproxen), 1.47 (d, 3, CH_3CH (lact)). ^{13}C NMR ($CDCl_3$): δ 173.8 (Cq), 170.6 (Cq), 157.4 (Cq), 135.6 (Cq), 134.9 (Cq), 133.8 (Cq), 133.5 (Cq), 132.6 (Cq), 129.1 (Cq), 128.7 (Cq), 128.4 (Cq), 126.8 (Cq), 126.2 (CH), 125.9 (CH), 118.6 (Cq), 105.4 (Cq), 68.9 (Cq, lact), 62.9 (CH_2), 55.1 (CH_3O), 45.0 (CH, naproxen), 18.5 (CH_3), 16.9 (CH_2, PMB), 16.8 (CH_3, PMB), 16.5 (CH_3, lact), 16.4 (CH_3, PMB). Anal. ($C_{29}H_{34}O_5$): C, H.

(c) Synthesis of Naproxen-L-Lactic Acid (cp. 1r). Deprotection was performed by the method of Steward et al.[36] A mixture of naproxen-L-lactic acid PMB ester (2.3 g, 5 mmol), anisole (12 mL), and TFA (10 mL) was kept for 2 min at room temperature. Thereafter excess reagent was removed under vacuum below 30 °C. The residue was dissolved in dichloromethane (100 mL) and washed with water (4 × 20 mL). The organic layer was extracted with diethyl ether (2 × 50 mL). Acidification with 6 N HCl gave the product, which was extracted with dichloromethane (4 × 25 mL). The washed and dried product (Na_2SO_4) was evaporated and the residue dried in vacuo at 50 °C. The product was crystallized from dichloromethane/cyclohexane. The yield was 75%. ^{1}H NMR ($CDCl_3$): δ 10.55–10.50 (br s, 1, OH), 7.54–6.93 (m, 6, aromatic), 4.97 (q, 1, $CHCH_3$ (lact)), 3.76 (q, 1, $CHCH_3$ (naproxen)), 3.69 (s, 1, CH_3O), 1.46 (d, 3, CH_3CH (naproxen)), 1.31 (d, 3, CH_3CH (lact)). ^{13}C NMR ($CDCl_3$): 176.1 (Cq), 173.6 (Cq), 157.3 (Cq), 135.0 (Cq), 133.5 (Cq), 129.1 (CH), 128.7 (Cq), 126.8 (CH), 126.1 (CH), 125.9 (CH), 118.6 (Cq), 105.4 (CH), 68.3 (CH, lact), 54.9 (CH_3O), 44.9 (CH, naproxen), 18.3 (CH, naproxen), 16.4 (CH_3, lact). IR (KBr): 1730 cm^{-1}. CI-MS: 301 [$(M - H)^-$]. Anal. ($C_{17}H_{18}O_5$): C, H.

(d) Synthesis of Naproxen-L-Lactic Acid NHS Ester. Naproxen-L-lactic acid (302 mg, 1 mmol) was dissolved in 10 mL of DMF. Thereafter DCC (277 mg, 1.1 mmol) was added. The solution was stirred for 15 min. NHS (115 mg, 1 mmol), previously dried at 50 °C in vacuo for 24 h, was added. The mixture was stirred for 24 h. The mixture was further treated as described for Nap-NHS. The yield was 91%. ^{1}H NMR ($CDCl_3$): δ 7.5–6.9 (m, 6, aromatic), 5.0 (q, 1, $CHCH_3$ (lact)), 3.8 (q, 1, $CHCH_3$), 3.7 (s, 3, CH_3O), 2.8 (s, 4, CH_2CH_2 (NHS)), 1.5 (d, 3, CH_3CH (naproxen)), 1.3 (d, 3, CH_3CH (lact)).

(e) Synthesis of Naproxen-L-Lactic Acid-Lysozyme (cp. 1s). Naproxen-L-lactic acid NHS ester (14.1 mg, 34.7 μmol) was dissolved in 10 mL of DMF and allowed to react with lysozyme (100 mg, 6.95 μmol) in a DMF/borate (0.025 M; pH 8.5) (20/80) mixture for 2 h and further treated as described under S7. The yield was 74%. The molar degree of substitution was 0.6, as established by the fluorimetric naproxen assay and Bradford protein assay.[32]

Z-Ala-Pro-Phe (cp. 6a). Z-Ala-Pro (44 mg, 0.14 mmol) was dissolved in 1 mL of DMF and DCC (30 mg, 0.14 mmol) was added. The solution was stirred for 15 min. Thereafter, NHS (17 mg, 0.14 mmol) was added and the solution was stirred overnight. The mixture was filtered and a solution of the NHS ester of Z-Ala-Pro (Z-Ala-Pro-NHS) was obtained. A 0.5-mL portion of this solution (corresponding with 0.07 mmol Z-Ala-Pro-NHS) was added to a solution of 24 mg (0.28 mmol) of phenylalanine in 0.5 mL of borate buffer (0.025 M; pH 8.5); 12.5 μL of TEA was added and the solution was stirred for 2 h. TLC showed a single spot and HPLC a single peak. The identity was verified by MS.

Leucine-Triamterene (cp. 9a-I, -II, -III). Triamterene (20 mg, 0.079 mmol) was dissolved in 2 mL of DMSO; BOC-Leu-NHS (111.6 mg, 0.47 mmol), dissolved in 0.5 mL of DMSO, and 120 μL of TEA were added. The mixture was stirred for 7 days. The DMSO was evaporated and the BOC group removed by addition of TFA (1 h, room temperature). The TFA was evaporated in vacuo and the residue was washed twice with 2 N NaOH. The residue was dissolved in DMSO. Purification was performed by reversed-phase HPLC. The three different product peaks were identified by LC-MS (Figure 3).

SM-aco-LYSO (cp. 10b). This coupling was done by the method of Shen et al.[28] for linking daunorubicin to poly(D-lysine). Sulfamethoxazole (202.5 mg, 0.799 mmol) was dissolved in 0.1 M sodium phosphate buffer (pH 8.7) (81 mL). The solution was cooled on ice and the pH was adjusted to 9.0–9.2 by the addition of 1 N NaOH. *cis*-Aconitic anhydride (682.75 mg, 4.37 mmol), dissolved in 15 mL of dry ACN (3-Å molecular sieves), was slowly added, while the pH was kept at 8.75–9.0 by addition of 1 N NaOH. The reaction was followed by TLC and HPLC. TLC ($BuOH/H_2O/HAc$ (35/10/5)): SM (R_f = 0.83), aco-SM (R_f = 0.57 (intense), 0.51), and aco(OH) (R_f = 0.22). RP-HPLC (ACN/H_2O/HAc (20/80/1) (system X)): SM (t_R = 6.1 min), aco-SM (t_R = 4.6 min), and aco(OH) (t_R = 3.0 min). The pH was adjusted to 7.4 by the addition of 1 N HCl. Unreacted SM was removed by elution on a gel-permeation column [Bio-Gel P-2 (fine, 2.5 × 30 cm); eluent PBS; flow 10 mL·min^{-1}]: SM (t_R = 17.4 min) and aco-SM (t_R = 10.0 min). The fractions (10 mL each) containing aco-SM were used for further coupling to lysozyme. The purity was established by TLC and HPLC (cp. **10a**). The solution of aco-SM (205 mg, 0.5 mmol) was placed on ice and the pH was adjusted to 5.0 by the addition of 0.1 N HCl. A freshly prepared solution of ECDI (0.96 mg, 5 mmol) in 25 mM sodium phosphate buffer (pH 5.0) was added. The pH of the reaction mixture was kept at 5.0 by the addition of 0.1 N HCl. After 5 min the temperature of the reaction mixture was raised to room temperature and the pH was elevated to 8.5 by the addition of 1 N NaOH. A freshly prepared solution of lysozyme (36 mg, 2.5 μmol) was added and the reaction mixture was stirred for 18 h. Thereafter undissolved protein was filtered off and the filtrate was passed through a Millex-GV (0.2 μm) filter and purified by gel-permeation chromatography on a Bio-Gel P-2 column or by several ultrafiltration steps (Amicon). The absence of SM and aco-SM was verified by HPLC of the protein solution and filtrate. The drug–protein conjugates were lyophilized and stored at −20 °C.

S1. Naproxen-Amino Acid Derivatives. Nap-NHS (14.0 mg, 0.043 mmol) was dissolved in 0.8 mL of 1,4-dioxane. The amino acid (0.043 mmol, Table I) was dissolved in 1.6 mL of borate buffer (0.025 M; pH 8.5) and the solution was stirred for 2 h. Thereafter, the mixture was acidified (0.1 N HCl) to pH 3–5 and extracted with dichloromethane; the organic layer was evaporated in vacuo, and the crude product was dissolved in the HPLC eluent and then purified by HPLC.

S2. Naproxen-Ester Derivatives. Nap-Cl (10.8 mg, 0.04 mmol) and glycolic acid (4 mg, 0.04 mmol), L-lactic acid (5 mg, 0.04 mmol), or tetra(L-lactic acid) (12.6 mg, 0.04 mmol) were

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dissolved in dry dichloromethane. TEA (11 μL, 0.08 mmol) was added and the mixture was stirred for 18 h. The reaction was followed by TLC. The ester derivatives were purified by reversed-phase HPLC.

S3. Indomethacin–Amino Acid Derivatives, Method 1. Indomethacin (2 g, 5.6 mmol) was dissolved in 30 mL of dry 1,4-dioxane (3-Å molecular sieves), and DCC (1.2 g, 5.8 mmol), freshly dissolved in 10 mL of 1,4-dioxane, was added and stirred for 15 min. Dried NHS (in vacuo, 70 °C) (0.8 g, 7.0 mmol), freshly dissolved in 10 mL of 1,4-dioxane was added and the solution was stirred for 24 h. The solution was filtered and a solution of the NHS ester of indomethacin (Indo-NHS) in 1,4-dioxane was obtained (0.112 M, solution A). The amino acid (0.067 mmol) was dissolved in 2 mL of 1,4-dioxane/borate buffer (0.025 M; pH 8.5) (20/80), and 0.5 mL of solution A (containing 0.056 mmol of Indo-NHS) was added, and the solution was stirred for 2 h. Products were identified and purified by HPLC.

S4. Indomethacin–Amino Acid Derivatives, Method 2. Indomethacin (120 mg, 0.34 mmol) was dissolved in 6 mL of dry 1,4-dioxane (3-Å molecular sieves) and DCC (70 mg, 0.34 mmol), freshly dissolved in 2 mL of 1,4-dioxane, was added. The solution was stirred for 15 min. Thereafter, S-NHS (73 mg, 0.34 mmol), freshly dissolved in 7 mL of 1,4-dioxane, was added. The solution was stirred for 18 h. The solution was filtered and a solution of the sulfo-NHS ester of indomethacin (Indo-S-NHS) was obtained (0.022 M, solution B). The amino acid (0.028 mmol) was dissolved in 2 mL of 1,4-dioxane/borate buffer (0.025 M; pH 8.5) (20/80), and then 1.25 mL of solution B (containing 0.028 mmol of Indo-S-NHS) was added and the solution was stirred for 2 h. Products were identified and purified by HPLC.

S5. Indomethacin–Amino Acid Derivatives, Method 3. Indomethacin (10 mg, 0.028 mmol) was dissolved in 2.5 mL of dry 1,4-dioxane (3-Å molecular sieves) and DCC (5.8 mg, 0.028 mmol), freshly dissolved in 0.5 mL of 1,4-dioxane, was added. This mixture was stirred for 15 min. After this time the amino acid (4.9 mg, 0.028 mol), previously dissolved in 1.5 mL of borate buffer (0.025 M; pH 8.5), was added and the solution was stirred for 18 h. Products were identified and purified by HPLC.

S6. Benzoic Acid–Amino Acid Derivatives. Benzoic acid (147 mg, 1.2 mmol) was dissolved in 5 mL of dried 1,4-dioxane (3-Å molecular sieves) and DCC (250 mg, 1.2 mmol), freshly dissolved in 0.5 mL of 1,4-dioxane, was added. The solution was stirred for 15 min. Thereafter, NHS (138 mg, 1.2 mmol), freshly dissolved in 0.5 mL of 1,4-dioxane, was added. The solution was stirred for 18 h. The solution was filtered and a solution of the NHS ester of benzoic acid (B-NHS) was obtained (0.4 M, solution C). The amino acid (0.2 mmol) was dissolved in 2 mL of 1,4-dioxane/borate buffer (0.025 M, pH 8.5) (20/80), and 0.5 mL of solution C (corresponding to 0.2 mmol B-NHS) was added, and the solution was stirred for 2 h at room temperature. Products were identified and purified by HPLC.

S7. Lysozyme Conjugates. Nap-NHS was dissolved in dioxane and allowed to react with lysozyme in a 1,4-dioxane/borate (0.025 M; pH 8.5) (20/80) mixture for 2 h. After filtration of the precipitated material, the filtrate was either dialyzed (spectropor; MW cutoff 5000–8000) for at least 9 days to remove unreacted noncovalently bound naproxen (checked by HPLC, Table IV) or subjected to gel filtration or ion-exchange chromatography. After a subsequent ultrafiltration using M-5 filters (Amicon) and lyophilization step the conjugate was stored at −20 °C.

Naproxen–spacer conjugates were prepared in an analogous manner; e.g. Nap–Leu-Gly-Gly-NHS was allowed to react under similar conditions.

Characterization of Drug–Protein Conjugates. The degree of drug substitution of protein conjugates was determined by quantitative analysis of protein and drug.

The protein content of the conjugates was determined by the method of Bradford[32] and A_{280} measurements.[33] Native lysozyme was used as standard, and appropriate corrections were made for present drug.

The drug content was assessed by HPLC, fluorimetric assays, or by measurements of reacted amino groups of proteins as described below.

HPLC Analysis of the Drug. Total drug present (sum of covalently bound and noncovalently bound drug) was assessed after alkaline hydrolysis of the naproxen–lysozyme conjugates. An exactly weighed amount of approximately 2 mg of lyophilized drug–protein conjugate was incubated with 1 mL of 6 N NaOH at 80 °C for 120 h. Thereafter the hydrolysate was placed on ice and acidified with 1.5 mL of 6 N HCl. Dichloromethane (6.0 mL) was added; the mixture was vortexed for 1 min and centrifuged at 3000*g* for 10 min. A 4.0-mL portion of the organic solvent was evaporated (nitrogen atmosphere, 60 °C). The residue was dissolved in HPLC eluent I (see Chromatography Section) (300 μL) and 100 μL was injected into the HPLC. Known amounts of naproxen and naproxen–lysine (1e) were routinely used as external standards.

Noncovalently bound naproxen was assessed by HPLC after drug extraction without a previous alkaline hydrolytic treatment. An exactly weighed amount of approximately 2 mg of lyophilized drug–protein conjugate was dissolved or suspended in 1 mL of water. A 1.5-mL portion of 1 M sodium phosphate buffer (pH 4.6) was added, 6.0 mL of dichloromethane was added, and the mixture was exactly treated and analyzed by HPLC as described above. Total drug present for SM-aco–LYSO conjugates was assayed after an acid hydrolytic treatment. Briefly 1 volume of the drug–protein solution (1 mg/mL), freshly dissolved in PBS, was mixed with 1 volume of 6 N HCl and the mixture was left to stand at room temperature for 2 h. Hydrolysis was completed within 30 min. Thereafter the hydrolysate was centrifuged (2 min; 3000*g*). The supernatant (100 μL) was injected into the HPLC. The column was a μBondapack C-18, the eluent was water/ACN/HAc (80/20/1 (v/v/v)), and the flow was 2 mL·min^{-1}. Calibration curves were constructed by adding known amounts of SM to native lysozyme. R^2 values were >0.999. Noncovalently bound SM was assayed after mixing 1 volume of the same solution with 2 volumes of acetonitrile. The samples were centrifuged for 2 min at 3000*g*. The supernatant (100 μL) was injected onto the HPLC column and further analyzed as described above.

Fluorimetric Drug Assay. Drug content was determined by a selective and quantitative fluorimetric measurement of naproxen. Fluorescence was measured at an excitation wavelength of 330 nm and emission wavelength of 360 nm (Figure 1). Calibration lines were constructed by adding known amounts of naproxen to native lysozyme solutions.

Number of Reacted Amino Groups of the Protein. Unreacted amino groups of the protein were estimated by the method of Habeeb.[16] From these data the number of reacted, i.e. drug substituted, amino groups was calculated (lysozyme contains seven amino groups).

Preparation of Male Rat Kidney Lysosomes. Male Wistar rats (250 g) were housed under a 12-h light/dark cycle in a temperature- and humidity-controlled room and were allowed to have free access to food and water.

Three rats were anesthetized with Nembutal (60 mg/kg, intraperitonially). The kidneys were perfused with ice-cold 0.3 M sucrose via the abdominal aorta. The perfused kidneys were removed, after which the cortices were excised, pooled (3 rats), weighed, and homogenized in 8 volumes of 0.3 M sucrose (4 °C). Renal cortices were homogenized in a Potter-Elvehjem glass homogenizer by 10 complete strokes with a Teflon pestle rotating at approximately 250 rpm. All subsequent steps were carried out at 4 °C. The crude homogenate was centrifuged at 143*g* for 10 min to sediment nuclei and unbroken tissue. A 0.5-mL portion of the homogenate was set aside for analysis of protein and acid phosphatase activity. Lysosomes were isolated from the cortical homogenate by differential centrifugation as described by Maunsbach.[34]

Briefly, nuclei and cell debris were removed by centrifugation at 143*g* for 10 min. The resulting supernatant was then centrifuged at 9500*g* for 3 min (excluding acceleration and deceleration). This step produced a three-tiered pellet which consisted of lysosomes (bottom layer), mitochondria (middle layer), and brush border (top layer). The brush border and mitochondria were removed by gently pipetting several milliliters of 0.3 M sucrose into the tube and resuspending the upper two layers. The suspension containing the rinse of brush border and mitochondria was discarded. The remaining lysosomal layer was resuspended in 0.3 M sucrose and centrifuged at 9500*g* for 3 min and the pellet was gently rinsed to remove any mitochondria. The remaining pellet was resuspended in 0.3 M sucrose buffer. The homogenates and lysosomal lysates were frozen and thawed 10 times in liquid

N_2 to lyse the organelles. Protein content was measured by the method of Bradford.[32] Acid phosphatase activity was determined with β-glycerophosphate as substrate by the method of Barrett.[35] Homogenates and lysosomal lysates were flash frozen in liquid N_2 and stored at -80 °C.

In Vitro Lysosomal Degradation Experiments. Conjugates or derivatives were incubated with homogenates or lysosomal lysates at different pH's. Conjugates and derivatives were dissolved in appropriate solutes. Incubations contained 250 μL of the homogenate or 100 μL of the lysate and were diluted with a 0.1 M phosphate buffer (pH 5.0 or 7.4) to a final volume of 1.0 mL. The final concentrations of the conjugates and derivatives based on parent compound present in the incubation media were as follows (μmol·L^{-1}): **1a–t**, 10; **2a–g**, 250; **3a–h**, 600; **4a**, **5a**, **6a**, 2000; **7a–b**, 75; **8a,b**, 4; **9a-I-III**, 4; **10a–b**, 70. The buffers contained a suitable internal standard for HPLC analysis (Chromatography Section). Samples (100 μL) were taken at various times and were diluted with either 2 volumes of ACN, 3 volumes of MeOH, or 1 volume of 2 N HCl to precipitate proteins. Samples were placed on ice for 15 min and then centrifuged at 3000*g* for 2 min. The supernatants were injected into the HPLC (Tables I and II). Peak ratios of derivative and parent compound were measured. The percentage parent compound generated, was calculated as

$$R(\text{PC, IS}) \times 100\% / (R(\text{Der, IS}) + R(\text{PC, IS}))$$

where R(PC, IS) and R(Der, IS) refer to the ratio (R) of the peak areas of the parent compound (PC) and the internal standard (IS) and that of the peak areas of the derivative (Der) and IS, respectively. In the case of protein conjugates, release was calculated as the percentage of drug released from the drug–protein conjugate (the initial total amount of drug present in the incubation media was set 100%).

Chromatography Section. TLC Analysis. TLC plates (silica gel 60F254, 10 × 5 or 20 × 20 cm) were from Merck (Darmstadt, FRG). Eluents were v/v(/v) MeOH/dichloromethane (50/50); BuOH/water/HAc (35/10/5); 1,4-dioxane/water/HAc (35/10/5), and ethyl acetate/hexane/HAc (33/66/1).

HPLC Analysis. The column was μ-Bondapack C-18. The following eluent systems and flow rates were used: I (a/b), water/ACN/concentrated HAc (60/40/1) at 1 (a) and 2 (b) mL/min; II, water/ACN/concentrated HAc (70/30/1) at 1.5 mL/min; III, phosphate buffer (67 mM, pH 7.0)/ACN (70/30) at 1.5 mL/min; IV, phosphate buffer (67 mM, pH 7.0)/ACN (60/40) at 1 mL/min; V, water/ACN/concentrated HAc (25/75/1) at 1.5 mL/min; VI, phosphate buffer (67 mM, pH 7.0)/MeOH (90/10) at 1 mL/min; VII (a/b), phosphate buffer (15 mM, pH 5.0)/ACN (80/20) at 1 (a) and 1.5 (b) mL/min; VIII, water/ACN/concentrated H_3PO_4 (85/15/0.2) at 2 mL/min; IX, water/ACN/concentrated H_3PO_4 (90/10/0.2) at 2 mL/min; X, water/ACN (60/40) at 2 mL/min; XI, phosphoric acid (10 mM, pH 3.2)/ACN (70/30) at 1.5 mL/min.

For the individual compounds the internal standards were (compound/internal standard) naproxen/sulfamethoxazole or 2-aminobenzoic acid, indomethacin/flurbiprofen, benzoic acid/salicylic acid and sulfamethoxazole/6-desmethylnaproxen.

Detection was performed by UV at 254 nm (indomethacin, benzoic acid, triamterene), 206 nm (Z-Ala-Pro, Z-Phe-Ala) or 260 nm (sulfamethoxazole) or by fluorescence (λ_{ex}, λ_{em}): naproxen (330, 360 nm), adriamycin (470, 560 nm), or triamterene (365, 440 nm).

FPLC. Pure soluble naproxen–lysozyme (1:1) was obtained by chromatography on the FPLC (Pharmacia). Mono-S HR 5/5 (Pharmacia) was used as cation exchanger. Proteins were separated by gradient elution: eluent A, 10 mM Tris HCl (pH 7.4); eluent B, 10 mM Tris HCl (pH 7.4) and 1 M NaCl. The flow was 1.0 mL/min and the eluent program was as follows: 0–5 min, 20% B, (sample application at 3 min); 5–15 min, 20% → 22.5% B; 15–19 min, 22.5% B; 19–20 min, 22.5% → 20% B.

On-line UV detection was performed at 280 nm for protein, and on-line selective fluorescence was done at excitation wavelength 330 nm and emission wavelength 360 nm for naproxen (Figure 2). Naproxen–lysozyme (0.5:1), stored at -20 °C, was dissolved in FPLC buffer (20% B). Undissolved protein was filtered off and the filtrate was passed through a Millex-GV (0.2 μm) filter, prior to injection into the FPLC. During each run, 2.5 mg of protein was applied to the column and 1.2 mg of pure soluble naproxen–lysozyme (1:1) was obtained (0.2 mg/mL).

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Potent and Systemically Active Aminopeptidase N Inhibitors Designed from Active-Site Investigation

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Derivatives of amino acids bearing various zinc-coordinating moieties (SH, COOH, CONHOH, and PO_3H_2) were synthesized and tested for their ability to inhibit aminopeptidase N (APN). Among them, β-amino thiols were found to be the most efficient with IC_{50}'s in the 11–50 nM range. These results suggest that the S_1 subsite of APN is a deep but not very large hydrophobic pocket, optimally fitting side chains of moderate bulk endowed with some degree of freedom. The iv administration of the inhibitors, alone, did not induce antinociceptive responses on the hot plate test in mice. However, in presence of 10 mg/kg acetorphan, a prodrug of the neutral endopeptidase inhibitor thiorphan, these compounds gave a large increase in the jump latency time with ED_{50}'s of 2 and 2.4 mg/kg for the disulfides of methioninethiol $[H_2NCH(CH_2CH_2SCH_3)CH_2S]_2$ and *S*-oxomethioninethiol $[H_2NCH(CH_2CH_2S(O)CH_3)CH_2S]_2$, respectively. These results show that the disulfide forms of β-amino thiols are efficient prodrugs of aminopeptidase N inhibitors capable of crossing the blood–brain barrier.

Introduction

The opioid peptides Met-enkephalin and Leu-enkephalin are degraded in the central nervous system by two zinc-metallopeptidases, neutral endopeptidase, NEP (EC 3.4.24.11)[1] and aminopeptidase N, APN (EC 3.4.11.2).[2,3] It has been proposed that inhibitors of both peptidases

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