Synthesis of Biotinylated and Photoreactive Probes for Angiotensin Receptors

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Three main types of bifunctional probe that can be obtained in a radiolabelled form and used for studying and purifying angiotensin receptors were developed: biotinyl-aminohexanoyl-[Tyr(3I)^4,-Phe(4N$_3$)^8]angiotensin II, iminobiotinyl-glycyl-aminohexanoyl-[Ala^1,Tyr(3I)^4,Phe(4N$_3$)^8]angiotensin II, and biotinyl-ethyl-1,3'-dithiopropionyl-[Ala^1,Tyr(3I)^4,Phe(4N$_3$)^8]angiotensin II. Several improved, unequivocal synthetic pathways are described for these products, using both 'stepwise' methods and a 'segment coupling' strategy allowing preparation of L-Phe and D-Phe derivatives (agonists and antagonists, respectively) from the same intermediate. Complete attribution of the ^{1}H NMR signals is reported. The molecules showed no folding in NOESY and temperature-variation experiments, suggesting that they are capable of interacting simultaneously with the receptor and with avidin. They all show an affinity of the order of 10^{-9} mol dm^{-3} for angiotensin II receptors. After introduction of the photoactivatable group, covalent bonding of the probe with the receptor was obtained with a mean yield of 25%. The biotin or related residue allows specific detection of the receptors among membrane proteins. This procedure can be applied to other receptors bearing biotinylation and photolabelling sites.

In spite of highly varied experimental approaches, such as the use of primary antibodies, anti-idiotypes, or complementary peptide, no membrane receptor of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, ANG II) has yet been directly isolated. The recent observation that the protein encoded by the oncogene *mas* has properties of an angiotensin receptor[1] has not solved the problem but rather has raised interest in developing a method for purifying this type of protein. The absence of conclusive results is perhaps due to the fact that no one has been able to propose solubilization conditions that provide a high yield while preserving receptor-binding capacities, unlike the case of other recently isolated receptors, *i.e.* those for insulin,[2] neurotensin[3] and luteinizing hormone.[4]

Based on the works of Hofmann with regard to the use of the avidin–biotin system,[5] and those of Guillemette and Escher concerning photoaffinity labelling of ANG II receptors,[6] we have developed a new concept of 'indirect affinity chromatography' (Fig. 1).

We describe the design, synthesis, characterization, and conformational ^{1}H NMR study of bifunctional probes used in the essential steps of our method of indirect affinity chromatography. These probes can of course be used in applications other than affinity chromatography, since they combine irreversible receptor binding with a biotinyl label that can be detected using a large number of commercial kits.[7]

Results

Synthesis of a Biotinylated, Photoactivatable Probe **6**.—In a preliminary work, we showed that ANG II can be substituted on its *N*-terminal portion without significant loss of its receptor-binding capacity or its hormonal biological effects. In this approach, biotinylation using a spacer arm gave the lowest nonspecific binding.[8]

In a second step, we were able to combine this modification with photoactivation of ANG II using a *p*-azidophenylalanine residue in position 8 of the hormone.[9] However, although this probe has been used to label a protein with all the characteristics of the receptor,[10] it still produced a perceptible level of nonspecific binding to succinoylavidin, which is used in the indirect affinity step.[5] We therefore designed a new probe, *i.e.*

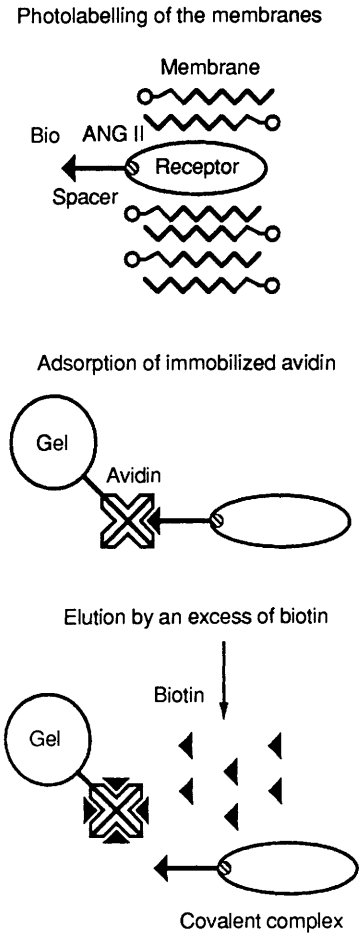


Fig. 1. Principle of indirect affinity chromatography. The receptor was first equilibrated with the probe and covalently bound by photolysis. Excess of probe and non-covalently bound probe were removed by washing. The membrane proteins are then solubilized with a suitable detergent (Triton X-100). The photobiotinylated receptor proteins were specifically adsorbed on immobilized tetrameric avidin. Lastly, the receptors were recovered by elution with a large excess of free biotin.

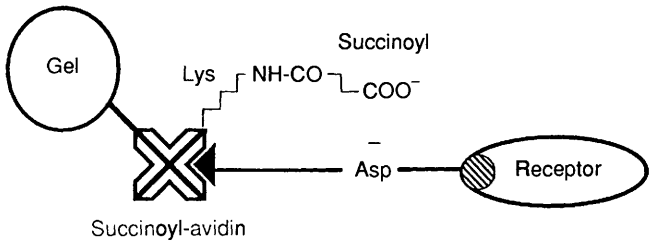
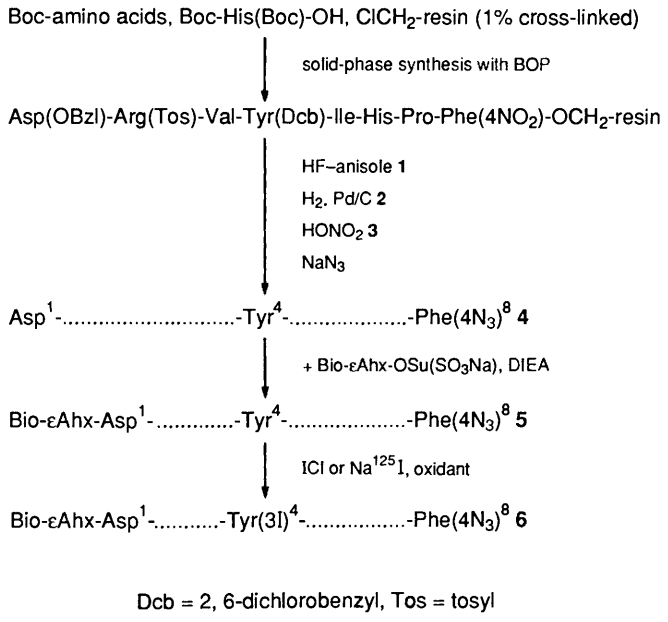


Fig. 2. Possible repulsion effect of the [Asp[1]]probe. Avidin has 9 positively charged Lys residues that can be succinoylated to give negative charges. Using the negatively charged Asp[1] derivative of ANG II it is hoped that nonspecific binding to avidin can be reduced.



Scheme 1. Synthetic pathway to the biotinylated probe **6**.

Bio-εAhx-[Asp[1],Phe(4N$_3$)[8]]ANG II,* which was expected to reduce the percentage of nonspecific binding significantly, because of opposition between its negative charge, borne by the β-carboxylic function of the Asp residue, and the negative charges of the succinoyl groups acylating the nine ε-amino-Lys functions of native avidin (Fig. 2). The importance of the carboxylic function in this position was recently illustrated.[11]

This probe could not be synthesized using the previously described strategy[9,12] because the lateral chain of Asp[1] is activatable, which precludes coupling of the previously used segments. We therefore resorted to 'salt coupling' of biotin to a pseudopeptide segment obtained by the method of Escher,[6] according to the synthetic pathway shown in Scheme 1, which is shown in much more detail in Scheme 3 and illustrated in Figure 3 for an analogous product (see later). All steps in the synthesis were optimized using HPLC monitoring. Only the synthesis of the final product of this study is described in detail. Table 1 gives chromatographic and UV characteristics of the main intermediates.

The structure of compound **5** was confirmed by ^{1}H NMR spectroscopy according to the procedure described for the last probe presented above. The main characteristics are given in Table 2. Compound **5** can be iodinated and photoactivated under conditions (not shown) reported for the Ala[1] probe[9] and has been shown by radioiodination to label a protein with the same characteristics.[10] However, its use in affinity chromatography does not appreciably decrease the percentage of nonspecific binding. Moreover, contrary to what was recently reported concerning the receptor of luteinizing hormone releasing factor (LRF) crosslinked to the ε-lysyl of a biotinylated probe, in which the receptors were recovered by elution with a solution containing 2 mmol dm^{-3} biotin,[13] we were unable to recover more than 10% in the case of angiotensin receptors. Significant recovery of the receptors could only be achieved by very drastic desorption methods, for instance using 70% formic acid, classical conditions for subsequent cleavage with BrCN, which entailed, however, a number of disadvantages, such as rupture of the bond between avidin and the gel, and avidin depolymerization.[14]

Table 1. HPLC properties of compounds involved in the synthesis of the biotinylated probe **6**.

Function Product	NO_2 **1**	NH_3^+ **2**	N_3 **-4**	Bio- **-5**	[I_1]Bio- **-6**	[I_2]Bio-
% Elution	36	26	36	41	43	45
$\varepsilon_{214}/\varepsilon_{254}$	5.4	63	2.1	2.2	3.3	3.3

Synthesis of an Iminobiotinylated Photoactivatable Probe **11**.—Owing to these difficulties in desorption of the [probe–receptor] complexes, we tried using several derivatives which are not so similar structurally to biotin. Several research groups have used Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril)[15] or chloramine-T (*N*-chlorotoluene-*p*-sulphonamide)[13] for radiolabelling of biotinylated products without any sign of deterioration in the biotin residue. Specific precautions for avoiding oxidation of this group have been described[16] whereas Iodogen has been proposed as an oxidizer for sulphydryl groups of proteins.[17] By rigorously monitoring the products by HPLC and NMR spectroscopy we found that oxidation easily occurred during iodination, even during times as short as one min, probably producing the sulphoxide of the biotin residue (biotinyl *S*-oxide = BioO). This increased the polarity of the products, resulting in decreased retention on the reversed-phase (RP)-HPLC column.[18] We have previously proposed using these oxidized derivatives as analogues which are structurally dissimilar to biotin.[8] Although a recent study on the streptavidin–biotin reaction suggested the possibility of an interaction between the sulphur of the heterocycle and the hydroxy group of the Thr[90] residue,[19] the ureido group is mainly responsible for the exceptional specificity of biotin for the glycoprotein avidin, and consequently a replacement of the S atom by Se,[20] or even its oxidation, does not appear to cause a sufficient decrease in affinity. Thus, like Finn and Hofmann,[5] we synthesized a probe containing an iminobiotin residue (denoted as Bin). In this derivative, the ureido group of biotin was replaced by a guanidino group, which is capable of binding avidin in its deprotonated form at pH 9.5–11, and detaching in its protonated form. Streptavidin is currently prepared by this method, which has also been proposed for the isolation of membrane proteins.[21] This group was thus grafted onto and ANG II derivative bearing a spacer arm, according to the method shown in Scheme 2.

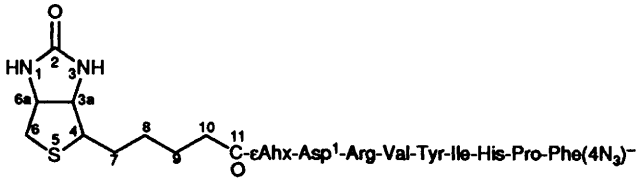
Synthesis of the intermediate **9**, using a three-level protection method very similar to that proposed by Neugebauer *et al.*,[22] has been described in detail.[12] It is easier to use the fluorenylmethoxycarbonyl group (Fmoc), of which a large number of derivatives are commercially available, than the nitrobenzyloxycarbonyl group {Z(NO$_2$)}, and it is entirely compatible with the presence of sulphur amino acids in the molecule, since it does not require hydrogenation. Table 3

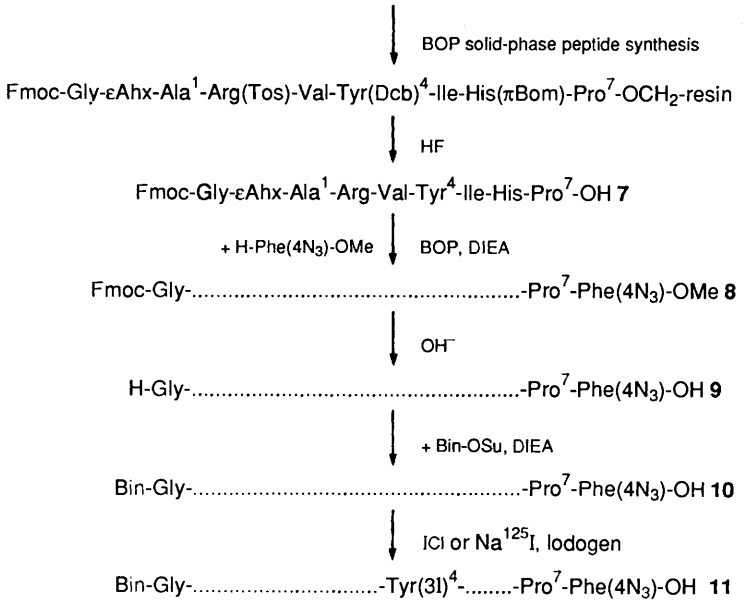
* Ahx = aminohexanoyl.

Table 2. ^1H NMR parameters (360 MHz), in [$^2H_6$]DMSO, of Bio-εAhx-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe(4N_3)-OH at 305 K. Chemical shifts are from the signal of residual [$^2H_5$]DMSO taken as reference at δ 2.5. Coupling constants are in Hz.

	NH			H_α		$H_{\beta\beta'}$				Others			
Residue		δ	^3J		δ	^3J		δ	^3J	^2J			δ	^3J
Asp[1]	d	8.13	7.5		4.54		β	2.63	4.9	16.3				
							β′	2.47	8.9					
Arg[2]	d	7.94	7.9		4.25		β	1.63			δδ′		3.03	
ε	t	7.45	5.6				β′	1.49			γγ′		1.43	
Val[3]	d	7.61	8.8		4.14		β	1.91			Me	d	0.77	7.3
											Me	d	0.74	7.3
Tyr[4]	d	7.98	8.4		4.49		β	2.82			$2H^{2,6}$	d	7.00	8.4
							β′	2.63			$2H^{3,5}$	d	6.61	8.4
Ile[5]	d	7.78	8.4		4.12		β	1.64			γMe	d	0.77	7.3
											δMe	t	0.78	
											γ		1.35	
											γ		1.05	
His[6]	d	8.29	7.4		4.75		β	3.03			C^2H	s	8.83	
							β′	2.90			C^4H	s	7.32	
Pro[7]					4.37		β	2.02			δ		3.64	
							β′	1.77			δ′		3.44	
											γγ′		1.81	
Phe[8]	d	8.30	7.8		4.42		β	3.02			$2H^{2,6}$	d	7.31	8.4
(4N_3)-OH							β′	2.92			$2H^{3,5}$	d	7.00	8.4
εAhx	t	7.31	5.6	$NHCH_2$	3.00	CH_2CONH		2.09 or 2.05			C^3H_2		1.2–1.6	
Bio-N^1H	s	6.34		H^{6a}	4.31		H^6	2.82			$C^{7,8,9}H_2$		1.2–1.8	
Bio-N^3H	s	6.39		H^{3a}	4.12		$H^{6'}$	2.57			$C^{10}H_2$	t	2.09 or 2.05	
	s			H^4	3.09									

Numbering Scheme for Bio-





Scheme 2. Synthetic pathway to the iminobiotinylated probe **11**.

summarizes the main chromatographic and spectral data of the synthesis.

Compound **10** was characterized by ^1H NMR spectroscopy using the two-dimensional homonuclear chemical-shift correlation (COSY) method (Table 4). As would be expected, the chemical shifts of the protons N^1H and N^3H in the heterocycle were clearly different in iminobiotinyl and biotinyl groups. A 2D nuclear Overhauser effect (NOESY) study only showed the proximity of the NH α groups of Val^3 and Arg^2 and the NH groups of His^6 and Ile^5, but showed no proximity between the hormonal portion of the molecule and its substituent, suggesting a linear structure. Compound **10** can be iodinated or radioiodinated, as previously reported, to give compound **11**.

Binding studies showed a high level of nonspecific binding, which makes the compound unusable without special precautions being taken.[14] The only significant difference from the preceding ligand is the guanidino group, which can interfere with plasma membrane. It is possible that the ligand could be used by masking this group with a suitable crown ether until a covalent bond is established,[23] and then removing it to allow trapping on avidin.

Table 3. HPLC properties of the compounds used in the synthesis of the iminobiotinylated probe **11**

Function	OSu	(N_3)	Bin-	$[I_1]$Bin-	$[I_2]$Bin-
Product	Bin-OSu	**9**	**10**	**11**	
% Elution	29	40	42	43	44
$\varepsilon_{214}/\varepsilon_{254}$	20	2.3	2.3	3.3	3.4

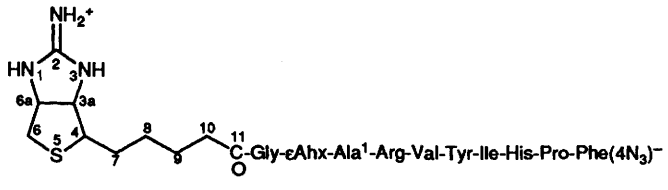
Synthesis of a Biotinylated, Photoactivatable Probe with a Cleavable Arm (Compound **17**).—We then considered inserting a disulphide bond between the hormonal portion of the probe and the biotin residue, which bond would be easy to cleave with an excess of thiol (dithiothreitol).[12] This strategy has been used successfully with other membrane proteins in the presence of detergents.[24] Purification of a membrane carboxylase of the Glu residue by affinity chromatography, with recovery by cleavage of an SS arm, has recently been reported.[25]

An initial molecule, Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$–CO–Gly-εAhx-[Ala^1,$Tyr(3I)^4$,$Phe(4N_3)^8$]ANG II, was synthesized.[12] Its particularly long spacer arm, with 19 bonds, was designed for use with whole cells, whose receptor sites are probably less accessible than those used in the preparation of purified membranes. This ligand, which may be adaptable to erasable labelling for use in cell sorting, has the disadvantage of showing relatively high nonspecific binding (30%). We therefore synthesized Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO-[Asp^1,$Tyr(3I)^4$,$Phe(4N_3)^8$]ANG II, which has a shorter spacer arm and is more hydrophilic (Seyer *et al.*, unpublished results). Lastly, based on

Table 4. ^1H NMR parameters (360 MHz), in [$^2H_6$]DMSO of Bin-Gly-εAhx-Ala-Arg-Val-Tyr-Ile-His-Pro-Phe($4N_3$)-OH at 305 K. Chemical shifts are from the signal of residual [$^2H_5$]DMSO taken as reference at δ 2.5. Coupling constants are in Hz.

	NH				H_α		$H_{\beta\beta'}$				Others			
Residue			δ	^3J		δ	^3J		δ	^3J	^2J			δ	^3J
Ala^1		d	7.97	7.1		4.25		CH_3	1.18	7.2					
Arg^2		d	8.05	8.0		4.24		β	1.66			δδ′		3.03	
	ε	t	7.77	5.8				β′	1.49			γγ′		1.35	
Val^3		d	7.51	8.8		4.17	6.1	β	1.92			Me	d	0.76	6.8
							8.8					Me	d	0.73	6.8
Tyr^4		d	7.98	7.9		4.50		β	2.82	4.5	14.5	$2H^{2,6}$	d_m	7.00	8.3
								β′	2.64	9.0		$2H^{3,5}$	d_o	6.61	8.3
Ile^5		d	7.80	9.0		4.13		β	1.64			γMe	d	0.76	6.8
												δMe	t	0.78	7.4
												γ		1.34	
												γ		1.03	
His^6		d	8.29	8.2		4.77		β	3.04			C^2H	s	8.92	
								β′	2.91			C^4H	s	7.35	
Pro^7						4.49		β	2.02			δ		3.64	
								β′	1.79			δ′		3.48	
												γγ′		1.79	
Phe^8		d	8.27	8.0		4.41		β	3.02			$2H^{2,6}$	d	7.31	8.4
$(4N_3)$-OH								β′	2.92			$2H^{3,5}$	d	7.00	8.4
Gly		t	7.96	6.0	CH_2	3.64									
εAhx		t	7.53	5.6	$NHCH_2$	3.08	CH_2CONH		2.09			C^3H_2		1.2–1.6	
Bin-N^1H		s	8.18		H^{6a}	4.65	4.9	H^6	2.92	5.0	13.2	$C^{7,8,9}H_2$		1.68–1.45	
							8.1								
Bin-N^3H		s	8.37		H^{3a}	4.46		$H^{6'}$	2.79	0.0	13.3	$C^{10}H_2$	t	2.13*	
Bin-C^2NH		s	7.72		H^4	3.25	4.5								
							5.6								
							8.3								

Numbering Scheme for Bin-



* Determined by NOESY studies.

Scheme 3. Synthetic pathway to the cleavable probe **17**.

the observation that protease V8 degrades most solubilized membrane proteins without affecting angiotensin receptors,[14] we considered the possibility of combining covalent labelling and affinity chromatography by using a step involving proteolysis with protease V8, which affects the peptide bonds close to acid residues (Glu or Asp).[26] We therefore synthesized Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO-[Ala^1,Tyr(3I)^4,Phe(4N_3)^8]ANG II (compound **17**) which can resist this treatment. It is the most promising ligand we have yet developed, which is why we describe its synthesis in detail.

We used the strategy proposed by Bodenmüller *et al.*,[27] with slight modifications (Scheme 3). Briefly, an ANG II derivative nitrated in the *para* position of the Phe residue was synthesized by classical solid-phase methods and was then hydrogenated to an amino derivative **13**, diazotized to an unstable diazonium salt **14**, and finally transformed into a photoactivatable azido derivative **15** by treatment with sodium azide. (i) Use of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium (BOP) reagent according to the 'rapid method' proposed by Le Nguyen *et al.*, with temporary protection of the lateral His chain in the form of Boc-His(Boc) (Boc = t-butoxycarbonyl),[28,29] allowed us to complete synthesis on resin in a single day, even working manually, with only 2 mol equiv. of each Boc-amino acid. The product was deprotected by HF and lyophilized overnight.

(ii) The high purity of the crude product made it unnecessary to carry out intermediate purification, which appears to be indispensable in other methods.[30,31] (iii) Hydrogenation was thus carried out directly in 0.1 mol dm^{-3} HCl (1 or 2 h) so that the diazotization step could be performed in the diluted, cooled hydrochloric solution just after removal of the catalyst. (iv) By monitoring each step by RP-HPLC with double UV detection (Fig. 3), the reactions could be rigorously checked. We were surprised to observe that the diazo derivative **14** was stable enough to be detected by HPLC without special precautions (Fig. 3c). It could be distinguished from the amino derivative **13** only by its extinction ratio $\varepsilon_{214}/\varepsilon_{254}$ (Table 5). This observation suggested that re-examination of the use of this type of derivative for labelling receptors and the development of appropriate conditions of photolysis might be of interest. Recent works show the advantage of conveniently substituted aryldiazo derivatives which operate through highly reactive carbenes (instead of nitrenes for arylazido derivatives).[23] (v) As recently reported,[30] when the reaction mixture is adjusted to a pH compatible with the RP-HPLC column, it can be loaded directly on the column after simple filtration. (vi) By routinely using a completely volatile eluant (0.1% CF_3CO_2H, v/v) and by screening fractions by analytical HPLC,[28] we can obtain a very pure product after simple lyophilization. This product is

Table 5. HPLC properties of the compounds used in the synthesis of the cleavable probe **17**.

Function	NO_2	NH_3^+	N_2^+	N_3	Bio-	$[I_1]$	$[I_1]$Bio-	$[I_1]$BioO-
Product	**12**	**13**	**14**	**15**	**18**	**16**	**17**	**19**
% Elution	33	25	26	38	43	44	49	43
$\varepsilon_{214}/\varepsilon_{254}$	5.4	60	3.3	2.0	3	3	3	

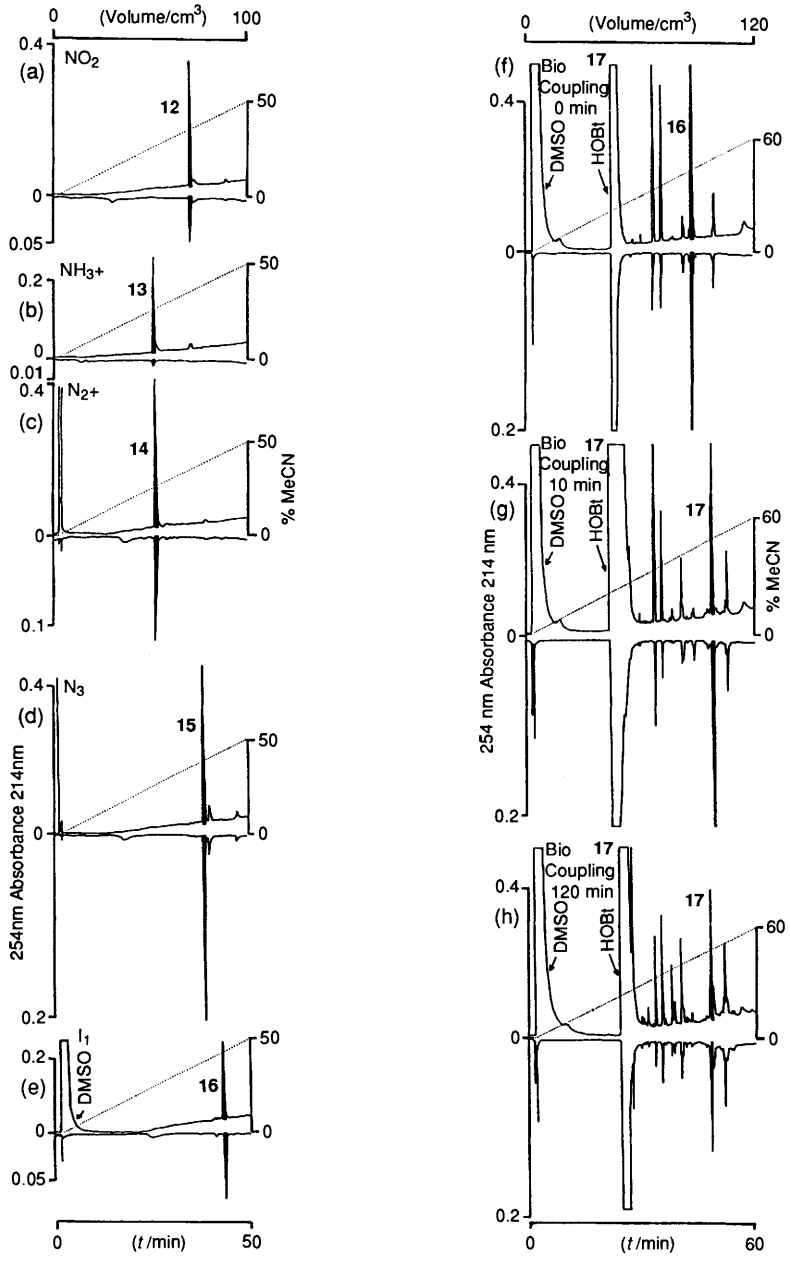


Fig. 3. HPLC monitoring of cleavable probe **17** synthesis. All reactions were monitored by RP-HPLC in a 0.1% (v/v) CF_3CO_2H-acidified system in the gradient mode (1% MeCN min^{-1}) from 0–50%, or to 60%, using dual-wavelength detection (214 nm up and 254 nm down). Flow 2 cm^3 min^{-1}; column C_{18} Lichrosorb, 4 × 250 mm, 5 μm (Merck). (a) The nitrophenylalanine derivative **12**, obtained by BOP solid-phase synthesis, can be used without purification. (b) Hydrogenation was achieved in 1–2 h. (c) The diazo derivative **14** produced a well resolved chromatographic peak without special care needing to be taken (at room temperature). It could be distinguished from the amino derivative **13** only by the $\varepsilon_{214}/\varepsilon_{254}$ ratio (the two compounds have the same positive charge). (d) The photosensitive derivative **15**, bearing the non-ionized azido group, is more lipophilic. Consequently, complete transformation of compound **14** can be checked. Compound **15** was isolated by semi-preparative HPLC on a 22 × 500 mm column (Whatman ODS 3, 10 μm). (e) ICl iodination of compound **15** gave a mixture of non-iodo **15**, monoiodo **16**, and diiodo derivatives in roughly equimolar amounts. An immediate semi-preparative step gave compound **16**, and compound **15** was recycled. Compound **16** was dissolved in DMSO and analysed. (f) The biotinylation reagent (NHS-SS-Biotin, from Pierce) and HOBt were added. The mixture was analysed before addition of base (DIEA), and showed the presence of unmodified compound **16**, DMSO, HOBt and, before eluting compound **16**, two peaks of partially hydrolysed biotinylation reagent. (g) Ten min after addition of base, an optimal proportion of the intended product **17** was present in the reaction mixture. (h) After 2 h under coupling conditions (pH 7–8), the product was partially degraded into many other azido compounds. The peak of compound **17** was contaminated by very similar side-products and was therefore very difficult to purify.

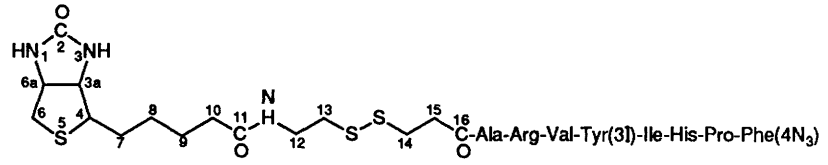
obtained in an ionization state (pH 2–3) that is often very favourable for the assignment of ^1H NMR signals.

ANG II has several iodination sites: positions 3 and 5 (*meta*) of the aromatic ring of Tyr[4], and positions 2 and 4 of the aromatic ring of His[6]. To allow the use of ^1H NMR spectroscopy in verifying the position of the iodine atom introduced into the monoiodinated derivative (unlabelled) and its homogeneity, we prepared a relatively large quantity of compound **17** (≈ 10 mg). Since the yield of the iodination step is inevitably limited to *ca.* 30%, we performed this reaction as early as possible (pathway A) to reduce the loss of a precious finished product, using an alternative pathway to that previously described.[9] By carrying

Table 6. ^1H NMR parameters (360 MHz) in [$^2H_6$]DMSO of Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO-Ala^1-Arg-Val-Tyr(3I)-Ile-His-Pro-Phe(4N_3)^8-OH at 305 K. Chemical shifts are from the signal of residual [$^2H_6$]DMSO taken as reference at δ 2.5. Temperature coefficients are in ppm $10^3 \times \delta_{NH}/\Delta T$ (ppb/K). Coupling constants are in Hz.

		NH			H_α		$H_{\beta\beta'}$		Others				
Residue	*T* coeff.		δ	^3J	δ	^3J		δ			δ	^3J	^4J
Ala^1	−3.2	d	8.16	7.2	4.28		Me	1.19					
Arg^2	−3.3	d	8.09	8.0	4.25		β	1.68	δ		3.08		
	−1.6 ε	t	7.45				β′	1.49	γ		1.61		
									γ		1.46		
Val^3	−2.5	d	7.50	9.0	4.17		β	1.94	Me	d	0.76	6.9	
									Me	d	0.73		
Tyr(3I)^4	−2.6	d	7.96		4.50		β	2.81	H^2	d	7.56	2.1	
							β′	2.60	H^5	d	6.74	8.2	
									H^6	dd	7.05	8.5	2.1
Ile^5	−5.1	d	7.87	8.5	4.14		β	1.65	γMe	d	0.78		
									δMe	t	0.79		
									γ		1.38		
									γ′		1.06		
His^6	−4.5	d	8.28	7.2	4.75		β	3.03	C^2H	s	8.7		
							β′	2.91	C^4H	s	7.3		
Pro^7					4.38		β	2.02	δ		3.64		
							β′	1.78	δ′		3.42		
									γγ′		1.83		
Phe^8	−3.7	d	8.31	8.0	4.42		β	3.04	$2H^{2,6}$	d	7.30	8.5	
(4N_3)-OH							β′	2.92	$2H^{3,5}$	d	7.00	8.5	
Spacer SS	NH	t	7.96	$NHCH_2$	3.31								
				CH_2SS	2.75								
				$SSCH_2$	2.86								
				CH_2CO	2.51								
BioN^{1}H	−3.3	s	6.34	H^{6a}	4.30		H^4	3.09	C^7H_2		1.61 and 1.46		
							H^6	2.82	$C^{8,9}H_2$		1.48–1.54		
Bio-N^{3}H	−4.2	s	6.38	H^{3a}	4.12		$H^{6'}$	2.58	$C^{10}H_2$	t	2.05	7.4	

Numbering Scheme for Bio-SS-



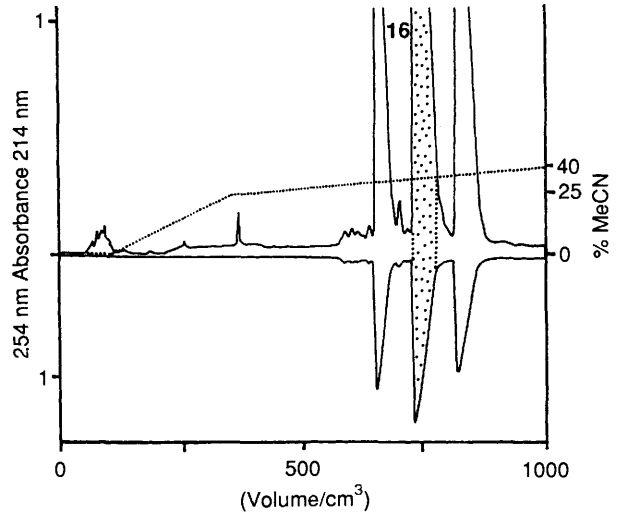


Fig. 4. Large-scale monoiodination of segment **15**. After 10 min of reaction with an equimolecular quantity of ICl in methanol, the mixture was diluted with 0.1% CF_3CO_2H in aq. solution and loaded onto the semipreparative column. The monoiodinated derivative **16** (stippled) was eluted at 10 cm^3 min^{-1} on a gradient whose slope was limited to 0.2% min^{-1} before consecutive desorption of the non-iodinated, monoiodinated and diiodinated derivatives.

out iodination with ICl in methanol [dimethyl sulphoxide (DMSO) did not provide satisfactory results, since it appeared to react with ICl], we overcame the problems of solubility inherent in the use of an aq. buffer and thereby prepared compound **16** in quantities as large as allowed by the RP-HPLC isolation step (Fig. 4). Biotinylation could be performed on the monoiodinated derivative **16**. We suspected that monoiodotyrosine might be much more susceptible to side acylations of its phenol OH group by sulphosuccinimide ester[32] because of the considerable decrease in pK induced by iodination (from 10.1 to 8.7).[33] For this reason, the coupling products were carefully analysed by HPLC. As an additional precaution, hydroxybenzotriazole was added to accelerate the procedure.[34] The reaction could be stopped at an optimal time to give a minimum of secondary peaks (Fig. 3g). By monitoring of the operation and use of the very specific extinction ratio of absorption at 214 and 254 nm introduced by the azido group, the desired product could always be identified without ambiguity, and was isolated in a single semipreparative RP-HPLC step.[9]

Structure of the Probe **17** *according to NMR Studies.*—In a COSY experiment,[35,36] the signals of the different spin systems, $HN\text{-}C_\alpha H\text{-}C_{\beta\beta'}H_2$, characteristic of each residue, were identified,[37] enabling us to determine the chemical shifts of the amide protons α, β, and sometimes β′, of all the amino acid residues. When the attribution remained ambiguous, such as in the case of the three ββ′ systems corresponding to aromatic residues

(His, Tyr and Phe), a study of the Overhauser effects (NOE) between residues, especially between the H_α of residue *i* and the N*H* of residue *i* + 1 (denoted by αN),[38–41] was carried out in a NOESY experiment.[42] The NMR spectrum was also used to confirm the substitution in the *para* position of Phe by the azido group (N_3) {two doublets, corresponding to the protons *meta* (H_m) or 3, 5, and *ortho* (H_o) or 2, 6}. In the case of the monoiodinated derivative, the three aromatic proton signals of Tyr (δ 7.56, s; 7.05, dd; 6.74, d) along with the Overhauser effect with the ββ′ protons in the case of two of them (including the singlet), showed that the iodine atom was located only in position 3, and excluded the possibility of iodination of His.

The signals of protons in the biotinyl heterocycle were easy to identify on the COSY map since they formed two characteristic spin systems, *i.e.* H_{3a} is coupled with N_3H and H_4, and H_{6a} is coupled with N_1H and $H_6H_{6'}$. The junction protons of the two rings are coupled to each other.

The different methylene groups of the cleavable arm, *i.e.* –NH–($C^{12}H_2$)–($C^{13}H_2$)–SS–($C^{14}H_2$)–($C^{15}H_2$)–CO–, were also identified by COSY. The signal of ($C^{12}H_2$), close to the nitrogen atom, appeared downfield from that of ($C^{13}H_2$), close to the sulphur atom. This was clearly verified, since the amide proton, whose signal is in triplet form, was coupled with ($C^{12}H_2$) (δ 3.31), which was itself coupled with ($C^{13}H_2$) (δ 2.75 ppm). Considering the electronegativity of sulphur and carbonyl, the signals of the two other methylenes ($C^{13}H_2$) and ($C^{15}H_2$) were expected to be in the same region. After attribution of all protons, two signals at δ 2.86 and 2.51, coupled only to each other, were attributed to ($C^{14}H_2$) and ($C^{15}H_2$). Based on an NOE with the amide proton of Ala, the signal at δ 2.51 was attributed to ($C^{15}H_2$). Incorporation of ε-aminohexanoic acid was confirmed by the triplet form of its amide proton signal (peptide bond with the carboxyl of biotin) and the doublet form of the amide proton of alanine. Chemical shifts and coupling constants are given in Table 6.

Conformation of the Probe according to NMR Studies.—To examine the conformation of the molecules, we measured the coupling constants ^3J(N*H*–C$_\alpha$*H*) and identified the NOEs observed on the NOESY map (not shown). The coupling constants, ranging from 7.0–9.0 Hz, indicated dihedral angles corresponding to an extended structure of the peptide portion of the molecule. This was verified by the fact that the inter-residues H_α–N*H* NOEs were stronger than the intra-residues. In the segment Ala^1 ··· His^6 of the monoiodinated derivative **17**, practically all $NH_i/H_{\beta\beta' i+1}$ NOEs were observed, as well as the αβ NOEs. The NOE between the H_α of His and the δδ′ protons of Pro was especially strong. Four N*H*/N*H* NOEs, *i.e.* Arg/Val, Val/Tyr, Tyr/Ile (weaker) and Ile/His, seemed to indicate that these amide protons are close to one another. However, these N*H*/N*H* NOEs were difficult to interpret conformationally, since: (i) the temperature coefficients did not indicate the presence of structurally stabilizing hydrogen bonds, and (ii) the coupling constants ^3J(N*H*–C$_\alpha$*H*) did not indicate any particular angular stress. No NOEs could be found between the biotin residue, the spacer arm, and the peptide portion. This could suggest that the probe is not folded; otherwise there could have been interactions between the different portions. It can therefore be concluded that the synthesized probes tend to adopt a linear structure, which is theoretically favourable for simultaneous binding with the receptor and with avidin.

Radioiodination of the Probe.—Small quantities of compound **15** were biotinylated to produce compound **18** (pathway B). This product can be iodinated or radioiodinated as previously described,[8] but this pathway has disadvantages. Even when Iodogen treatment of the probe was shortened as much as possible (only 1 min), at least a fourth of the labelled product was lost in the form of the *S*-oxide of the monoiodinated derivative. Moreover, the variability of the NaOH content in radioactive iodine preparations (IMS 300, Amersham) makes this operation very unreliable because of the influence of pH on the oxidation rate of the biotinyl residue. Furthermore, purification of the unoxidized monoiodinated derivative is relatively difficult due to: (i) the nearness of its MeCN elution percentage and that of its *S*-oxide, and (ii) coelution of the non-iodinated derivative **18** with the *S*-oxide **19** of the probe **17** (Table 5). On the other hand, biotinylated and non-biotinylated derivatives are eluted at MeCN concentrations differing by 5–6% (Table 5). Owing of this, pathway A is preferable for radiolabelling of the probe. Iodine can thereby be introduced with the normal yield obtained when radiolabelling a large excess of angiotensin, *i.e.* with formation of only trace amounts of diiodinated derivative. The purified radiolabelled product is then coupled with an excess of biotinylation reagent under mild non-oxidizing conditions, which we carefully explored (Fig. 3), to provide a high yield of the radiolabelled probe. This procedure is related to that of Bolton, who proposed labelling by coupling rather than by oxidation.[43] It offers the additional advantage of allowing total incorporation of radioactivity, a large quantity of which is often lost in the iodinated Bolton–Hunter reagent *N*-[3-(4-hydroxyphenyl)propionyloxy]succinimide, because it is bound to a hydrolysed portion of the active ester, which cannot be incorporated. The availability of a properly characterized batch of monoiodinated derivative for use as a chromatographic reference standard facilitates its identification by HPLC and allows isotopic dilution of the same radioactive product labelled in large scale affinity chromatography.

Care in the Use of the Cleavable Probe **17**: *Destruction of the Azido Group by Thiols.*—Destruction of the azido group by reducing agents such as thiols, which may be used in binding and protein-purification procedures, is well documented.[44] We verified by RP-HPLC that Phe($4N_3$), incubated in the presence of dithiothreitol (DTT), is transformed into a polar species, as indicated by a clear decrease in retention time, particularly in neutral or basic medium (not shown). It probably corresponds to amine protonated in the HPLC eluant (pH 2.2).

Fragility of the Disulphide Bridge.—We observed rapid destruction of the probe under coupling conditions with the active ester of sulphosuccinimide (Fig. 3h). This could arise from two main causes: (i) parasitic coupling on unprotected lateral chains of angiotensin, particularly the monoiodotyrosine residue [Tyr(3I)], and His or Arg; (ii) cleavages and exchanges of the disulphide bridge under nearly basic conditions.[44] The extreme scarcity of the covalent [probe–receptor] complex compared with other membrane proteins (of the order of 10^{-5}) increases the chances of an irreversible exchange of the dissymmetric disulphide bridge with these proteins,[44] which frequently have a high cysteine content.

Discussion

In the present article we describe the three essential steps in our strategy for developing pseudo-peptide probes used in purifying angiotensin receptors by indirect affinity chromatography. Our first concern was to test the validity of the procedure by applying it to analytical purification. We were among the first groups to propose combining photoaffinity labelling with biotinylation to isolate a membrane protein.[10] A related approach was carried out in parallel for insulin,[45] whose receptor was one of the first to be purified by affinity chromatography with the avidin–biotin system.[5] However, the modifications of this high-molecular-weight peptide hormone were much more critical than those of ANG II, which is only an octapeptide. Hofmann

et al. succeeded in combining biotinylation (using dethiobiotin) and crosslinking in a corticotropin (ACTH) analogue comprising 24 residues.[46] Hazum *et al.* managed only to alternate photoactivation and biotinylation on analogues of LRF,[47] which is a decapeptide, whereas Bladon combined biotinylation and crosslinking.[13] In the analytical step, it was indispensable for these modifications to be co-ordinated with radiolabelling showing high specific activity, produced by monoiodination of the Tyr residue of ANG II.

Based on observations of insulin receptors in human placenta and rat liver,[2] it was expected that the spacer arm would play a very important role in affinity chromatography, and consequently we first tested spacer arms with widely differing structures. In the case of ANG II, it appeared that the length of the spacer arm does not have a very crucial effect on the affinity of the probe for the receptor, since substituents as different as Bio-εAhx-,[9] Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO-Gly-εAhx,[12] and Bio-εAhx-Gly-εAhx- (Seyer *et al.*, unpublished results) differed by less than one order of magnitude. Moreover, to a first approximation, its length did not appear to have a very large effect on binding to avidin, beyond a certain size that we first limited to that of Bio-εAhx, based on the results of other authors.[5] The only appreciable effect was on the level of nonspecific binding.

On the other hand, we were obliged to test several methods for recovery of covalent [probe–receptor] complexes, despite the force of the avidin–biotin interaction. The use of iminobiotin has not been particularly well explored because of its high level of nonspecific binding to liver membranes. We considered that this problem could be overcome by temporarily masking its guanidyl group until the covalent bond was formed. Insertion of a cleavable spacer arm with a disulphide bridge is an attractive solution, but the fragility of the molecule calls for many precautions during the synthesis and use of this type of ligand, including the following: (i) basic media must be avoided since they favour the exchange of disulphide bridges, (ii) free thiols must be avoided, which is not always compatible with receptor binding, (iii) the radiolabelling method has to preserve the integrity of both the disulphide bridge and the biotinyl group.

Specific high-yield synthetic methods of producing these products were tested. We were able to work on a larger scale than in previous studies, and to shorten synthesis by eliminating many steps, particularly purifications. Synthesis could be optimized by routine use of HPLC with double UV detection. Similarly, isolation of the products by HPLC consistently assured a high degree of purity. We thereby detected the oxidation of the biotin residue during radiolabelling with Iodogen, which, to our knowledge, has not previously been reported.

The probes were completely characterized by NMR spectroscopy. Of all the methods tested, *i.e.* amino-acid analysis, biological activity, light absorption, and reactions characteristic of the biotinyl group, ^{1}H NMR spectroscopy was the most effective and reliable, since it is applied to the intact, recoverable molecule. The hydrolytic step required before any amino-acid analysis results in deiodination,[33] together with deterioration of the unstable azido group in strongly acidic medium. Consequently, many residues which compose the molecule are not routinely detectable by this method (aminohexanoic acid, azidophenylalanine, biotin, iminobiotin).

New probes are being investigated in order to facilitate the preparative step in the purification of angiotensin receptors. Use of more refined analogues and optimization of the spacer arms should decrease the level of nonspecific binding. There are several possible pathways that could increase the rate of covalent binding using new ligands (80% has been obtained in certain cases):[25] (i) use of azido groups activated by a nearby NO_2 group (Eberlé and de Graan[44]), (ii) use of other photoactivation sites, (iii) use of diazonium salts for photoactivation,[23] (iv) specific activation of the β-carboxylic function of Asp^1, (v) crosslinking of the probe, (vi) formation of a disulphide bond with the receptor,[48] and combinations of several of these pathways.

Conclusions

The tools described here appear to be well adapted to the study of angiotensin receptors, allowing new approaches to their localization, isolation, and internalization, as well as to their structure, since they very specifically label the binding site of the hormone, and make it possible to sort labelled fragments from the rest of the membranous peptide material. Sequencing of fragments modified by photoaffinity[49] is an effective way of studying the binding site of the hormone, which is extremely important for the rational development of antagonists. These probes now form the basis of a procedure for analytical purification of the angiotensin receptor from various tissues by affinity chromatography. This purification has ben reported many times but never confirmed or developed. As expected, the cleavable arm of the described probe permitted us to effect easy receptor recovery. The DTT-liberated thiol group could be used for a supplementary purification step using Thiopropyl-Sepharose (Pharmacia).[14]

The recent identification of the oncogene *mas* with an angiotensin receptor suggests that its overexpression could be achieved in cell systems by molecular biology methods. Hence, this enriched and abundant material might provide a means for overcoming the great remaining obstacle in the study of receptors occurring naturally in animal tissues, *i.e.* the scarcity of material. Moreover, the combination of photoaffinity labelling with methods of affinity chromatography should provide solutions to many other problems that occur in the study of other receptors, and more generally other macromolecules of biological interest (enzymes, regulation factors, nucleic acids). The method can immediately be applied to a certain number of other receptors on which independent photoactivation and biotinylation sites have been identified in preliminary studies. The development of specific methods for the total synthesis of these types of probe is also an important objective of these studies, since it is indispensable that they be prepared in large quantities, if only to verify their structure.

Experimental

Chemicals.—Bio-εAhx-OSu (NHS-LC-Biotin), Bin-OSu·HBr (NHS-Iminobiotin), Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO–OSu(SO_3Na) (NHSS-SS-Biotin) and Iodogen were purchased from Pierce. MeCN for HPLC was UV-grade (Carlo Erba). Spectroscopic-grade anhydrous DMSO (Fluka) was used for segment couplings and HPLC separations. During coupling, pH was evaluated in organic solvents using a moistened paper indicator (Prolabo). Diazo and azido derivatives were handled in dim light. The stoicheiometries of the counter-ions (mainly CF_3CO_2H) of the different products were not determined.

High-performance Liquid Chromatography.—A Lichrosorb C_{18} column (5 μm; 4 × 250 mm, from Merck) with a 2 cm^3 min^{-1} flow rate was used in analytical studies, and the preparative runs were done on a Partisil ODS 3 Magnum 20 column (10 μm; 22 × 500 mm, from Whatman). As often as feasible, the entirely volatile CF_3CO_2H–water–MeCN eluant system was used, with 0.1% CF_3CO_2H (v/v). Eluants and samples dissolved in both aq. and organic solvents were filtered over regenerated cellulose membranes (porosity 0.45 μm, Sartorius). Linear gradients were used in all cases. Percentages of MeCN given in the Tables and HPLC diagrams

correspond to effluent flowing through the detector cell at the time of UV detection (corrected for the dead volume of the HPLC system, v_0). They are given as an indication only, since the absolute value depended on the type and condition of the column used. The ratios of UV extinction are also given as an indication, as evaluated using the photometers of the HPLC system.

Amino-acid Determination.—Hydrolysis was carried out in the dark in an evacuated sealed ampoule using 6 mol dm^{-3} HCl containing 1% phenol (for recovery of Tyr), for 21 h at 110 °C. Amino acids were analysed by automated precolumn derivatization with phthalaldehyde (Waters system). Pro, εAhx, Phe($4N_3$) and Bio were not quantified in this way.

Nuclear Magnetic Resonance.—All spectra were recorded at 305 K with a Bruker WM 360 WB spectrometer operating at 360 MHz for protons, interfaced with an Aspect 3000 computer. The sample (8 mg, usually at a pH of *ca.* 3, as obtained by lyophilization of HPLC fractions) was dissolved in [$^2H_6$]-DMSO (99.8% deuteriated; 0.5 cm^3). The residual ^1H DMSO peak was taken as the reference at δ 2.5. Correlations between protons coupled in a scalar manner were determined in a 2D experiment using the sequence D_1–90°–D_2–45°–acquisition (COSY). Spectra were recorded in 512 increments of D_2. Processing was carried out with 2 × 1024 data points in F_2 and 1024 in F_1. Before calculating the Fourier transform, free-induction decays (FID) were multiplied by a non-shifted sine-bell window function in both domains.

Correlations between protons coupled in a dipolar manner (NOESY) were obtained using the sequence D_1–90°–D_0–90°–τ_m–90°–acquisition in the phase-sensitive mode with a mixing time τ_m of 350 ms in 256 increments.[42] Before calculating the Fourier transform, the FID were multiplied by a sine-bell function shifted by π/4 in F_1 and π/8 in F_2.

Temperature coefficients were obtained from four spectra recorded at 32, 37, 42 and 47 °C.

Synthesis of [*Asp*^{1},*Phe*($4NO_2$)^8]*ANG II* **1**.—Synthesis was performed with BOP as previously described until Arg^2 incorporation,[28] as shown in Scheme 1. An aliquot (1 g) of peptidyl resin was acylated with Boc-Asp(OBzl)-OH, *N*-deprotected, and totally deprotected according to the classical HF–anisole procedure to give compound **1**, which was lyophilized (605 mg).

Synthesis of [*Ala*^{1},*Phe*($4NO_2$)^8]*ANG II* **12**.—Another aliquot (1 g) of peptidyl resin, acylated with Boc-Ala-OH, gave compound **12** (598 mg).

Synthesis of CF_3CO_2H·*Bin-Gly-*ε*Ahx-*[*Ala*^{1},*Phe*($4N_3$)^8]-*ANG II* (CF_3CO_2H·**10**).—Compound **9**, obtained as previously described[12] (14 mg), was added to an excess of Bin-OSu·HBr (20 mg) in anhydrous DMSO (500 mm^3). The reaction was monitored by RP-HPLC in the gradient mode. Diisopropylethylamine (DIEA) was added to maintain pH at 8–9. Coupling was complete after 1h. The mixture was reacidified, and purified by preparative RP-HPLC.[9,12]

Synthesis of CF_3CO_2H·[*Ala*^{1},*Phe*($4N_3$)^8]*ANG II* (CF_3CO_2H· **15**).—Crude peptide **12**, obtained after HF deprotection and lyophilization, was dissolved in 0.1 mol dm^{-3} HCl (300 mg per 50 cm^3), resulting in a yellowish solution, which was hydrogenated at atmospheric pressure by bubbled H_2 in the presence of a catalyst (10% Pd/C; 200 mg). The reaction was complete after 2 h (Fig. 3b). The catalyst was removed by filtration on Celite, which was then rinsed with 0.1 mol dm^{-3} HCl (to a total volume of 150 cm^3). To an aliquot of solution cooled to 0 °C (50 cm^3, corresponding to 100 mg of crude peptide) were gradually added the following during 5 min: 1 mol dm^{-3} $NaNO_2$ (1 cm^3, ~15 mol equiv.) followed by 1 mol dm^{-3} urea (1 cm^3, 15 mol equiv.). An aliquot of medium (15 mm^3) was then removed and frozen for HPLC analysis (Fig. 3c). Immediately afterward, 1 mol dm^{-3} NaN_3 (1 cm^3, 15 mol equiv.) was added in the dark and the medium was first agitated for 30 min at 0 °C, and then agitated under a slight vacuum for 30 min with gradual return to room temperature. HPLC analysis showed the total disappearance of compounds **13** and **14** after *ca.* 15 min (Fig. 3d). The pH was adjusted to a value compatible with a C_{18} column (~3) by using 1 mol dm^{-3} NaOH (2 cm^3), and the solution was filtered (0.45 μm), and then loaded onto the semi-preparative column. The main peak of the azido derivative **15**, which was located using the analytical chromatogram of the reaction medium (Fig. 3d), was divided into fractions, each of which was examined by isocratic analytical HPLC (30% MeCN).[28] The purest fractions were combined and lyophilized, with emphasis on purity rather than yield (136 mg of CF_3CO_2H· **15** from 300 mg of crude product).

N.B.: Owing to the high concentrations of azido derivative in the eluate and the high flow rate, the fraction of product photolysed in the HPLC detector cell was negligible.

Iodination. Synthesis of CF_3CO_2H·[*Ala*^{1},*Tyr*(3I)^4,*Phe*-($4N_3$)^8]*ANG II* (CF_3CO_2H·**16**).—Compound **15** was dissolved in MeOH (20.8 mg in 1 cm^3). 0.1 mol dm^{-3} ICl in MeOH (180 mm^3; 1 mol equiv.) was added portionwise at 0 °C. After 10 min, the mixture was diluted with 0.1% (v/v) CF_3CO_2H in water and purified by HPLC (Fig. 4). After lyophilization, the second peak gave compound **16** (~8 mg).

Biotinylation. Synthesis of CF_3CO_2H·*Bio*-NH[CH_2]$_2$–SS–[CH_2]$_2$CO-[*Ala*^{1},*Tyr*(3I)^4,*Phe*($4N_3$)^8]*ANG II* (CF_3CO_2H· **17**).—The following were added to compound **16** (1 mol equiv.) (9 mg; ~9 μmol): an aliquot of Bio-NH[CH_2]$_2$–SS–[CH_2]$_2$CO–OSu(SO_3Na) (16 mg, ~3 mol equiv.), a catalytic amount of hydroxybenzotriazole (HOBt) hydrate (2 mg),[34] and DMSO (0.5 cm^3). The mixture was stirred for 10 min and an aliquot (15 mm^3) was analysed by HPLC (Fig. 3f). DIEA was then added to maintain pH at *ca.* 9 (20 mm^3). After 10 min (Fig. 3g), the mixture was reacidified and compound **17** was isolated by HPLC.

Synthesis of CF_3CO_2H·[*Asp*^{1},*Phe*($4N_3$)^8]*ANG II* (CF_3CO_2H·**4**).—Compound **4** was obtained from compound **1** as described for the preparation of compound **16**.

Biotinylation. Synthesis of CF_3CO_2H·*Bio-*ε*Ahx-*[*Asp*^{1},*Phe*-($4N_3$)^8]*ANG II* (CF_3CO_2H·**5**).—Compound **4** (16 mg, ~16 μmol) and Bio-εAhx-OSu(SO_3Na) (~16.5 mg, 30 μmol, 2 mol equiv.) were dissolved in DMSO (500 mm^3). The medium was maintained at pH 7–8 with DIEA (50 mm^3) for 2 h. The medium was reacidified (pH 3) with CF_3CO_2H and the product was isolated by semi-preparative HPLC.

Iodination. Synthesis of CF_3CO_2H·*Bio-*ε*Ahx-*[*Asp*^{1},*Tyr*(3I)^4,*Phe*($4N_3$)^8]*ANG II* (CF_3CO_2H·**6**).—Iodination was performed with ICl on small quantities (<1 mg) in a buffered aq. medium, and radiolabelling was performed with $Na^{125}I$ and Iodogen as previously described.[8]

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