Supplementary Material (ESI) for Chemical Communications

Merging homogeneous catalysis with biocatalysis. Papain as hydrogenation catalyst.

Lavinia Panella,[a] Jaap Broos,[b] Jianfeng Jin,[b] Marco W. Fraaije,[b] Dick B. Janssen,[b] Margot Jeronimus-Stratingh,[c] Ben L. Feringa,[a] Adriaan J. Minnaard[a] and Johannes G. de Vries*[a,d]

[a]*Department of Organic & Molecular Inorganic Chemistry and* [b]*Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands.*
[c]*Faculty Centre for Mass Spectrometry, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.*
[d]*DSM Research, Life Sciences-Advanced Synthesis & Catalysis, P.O. Box 18, 6160 MD Geleen, The Netherlands*

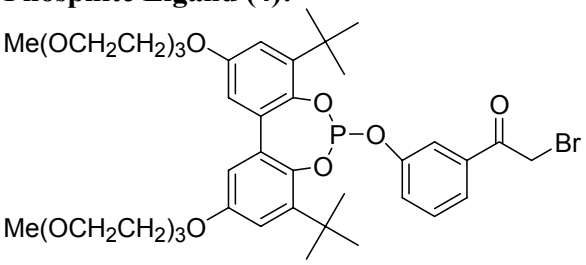
Hans-JG.Vries-de@dsm.com

Experimental section

General remarks

For synthetic purposes, solvents were reagent grade, dried and distilled before use following standard procedures. Dioxane was distilled over sodium under nitrogen or purified over neutral alumina and degassed; buffer solutions and water were degassed for 2-3 hours prior to use. Papain was obtained from Sigma-Aldrich as a buffered aqueous suspension in 0.05 M sodium acetate (1.1 mM). *N*-Z-glycine *p*-nitrophenyl ester was purchased from Bachem. DTT (dithiothreitol) was obtained from Roche.
^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were recorded at room temperature in $CDCl_3$ on a Varian Mercury-Plus 400 MHz spectrometer. Chemical shifts were determined relative to the residual solvent peaks ($CDCl_3$, δ = 7.26 ppm for proton, δ = 77 ppm for carbon) and external H_3PO_4 for ^{31}P-NMR. Data are reported as follows: chemical shift (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz) and integration. Mass spectra were recorded on an AEI-MS-902 mass spectrometer. UV-Vis measurements were performed on a Hewlett-Packard HP 8453 FT spectrophotometer. Enantiomeric excesses were determined by capillary chiral GC analysis on a HP 6890 gas chromatograph equipped with a flame ionization detector.

Phosphite Ligand (4):



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2-Bromo-1-[3-(4,8-di-*tert*-butyl-2,10-bis-{2-[2-(methoxy-ethoxy)-ethoxy]-ethoxy}-5,7-dioxa-6-phopha-dibenzo[*a,c*]cyclohepten-6-yloxy)-phenyl]-ethanone.

PCl_3 (160 µL, 1.1 eq) was added dropwise at 0 °C to a solution of dry toluene (5.0 mL) containing NEt_3 (0.9 mL, 4 eq). In the mean time, bisphenol **3** (1.0 g, 1.6 mmol, 1 eq) was dried by repeated addition and removal under reduced pressure of toluene (5 mL, 3 times) and then dissolved in toluene (10 mL) and added dropwise to the reaction mixture at 0 °C. The reaction mixture was allowed to reach room temperature and stirred for 3 h. After which time dry Et_2O (5 mL) was added and the salts quickly filtered, the resulting solution was then concentrated under reduced pressure. Toluene (5 mL) was added to the syrup obtained, followed at 0 °C by NEt_3 (0.9 mL, 4 eq). A solution of 3-hydroxy-phenacyl bromide (343.6 mg, 1.6 mmol, 1 eq) in toluene (10 mL) was then added dropwise, the reaction mixture was allowed to reach room temperature and stirring was continued for further 3 h. After which time, Et_2O (5 mL) was added and the salts quickly filtered over a plug of silica. The resulting solution was concentrated under reduced pressure and the crude mixture was purified by flash column chromatography on silica gel (ethyl acetate/heptane 3:2) yielding the desired product in 72% yield as a colorless syrup, usually store at -12 °C.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J*= 8.0 Hz, 1H), 7.58 (s, 1H), 7.41 (t, *J*= 8.0 Hz, 1H), 7.28 (d, *J*= 8.0 Hz, 1H), 7.04 (d, *J*= 3.2 Hz, 2H), 6.72 (d, *J*= 3.2 Hz, 2H), 4.35 (s, 2H), 4.17-4.11 (m, 4H), 3.89-3.85 (m, 4H), 3.79-3.72 (m, 4H), 3.72-3.63 (m, 8H), 3.58-3.52 (m, 4H), 3.37 (s, 6H), 1.45 (s, 18H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 190.4 (s), 155.2 (s, 2C), 152.5 (s), 142.6 (s, 2C), 141.2 (s), 135.3 (s, 2C), 133.5 (s, 2C), 130.1 (d), 126.0 (d), 124.3 (d), 120.6 (d), 115.3 (d, 2C), 113.6 (d, 2C), 71.9 (t, 2C), 70.8 (t, 2C), 70.64 (t, 2C), 70.56 (t, 2C), 69.7 (t, 2C), 67.8 (t, 2C), 59.0 (q, 2C), 35.4 (t), 31.0 (q, 3C), 30.9 (q, 3C), 30.8 (s, 2C). ^{31}P-NMR (162 MHz, $CDCl_3$) δ 137.1. MS for C42H58BrO12P, *m/z (%)*: 864 (M^+, 0.5%), 786 (100%); HRMS (EI^+), for C42H59O12P calculated on 786 (M^+-Br): 786.3748, found: 786.3743.

Alkylation of papain (5)

An aliquot (100 µl) of papain suspension was dissolved in phosphate buffer (9.8 ml, 100 mM, pH 7) containing DTT (100 µl, 100 mM) as reducing agent.[1] The incubation was performed under nitrogen atmosphere at 25 °C for 20 min, while gently stirring. Subsequently, a solution of **4** in dioxane (2 mM) was added in portions (6 × 0.5 ml) over a period of 3 h to ensure high degree of modification of the protein, while gently stirring at 25 °C. The mixture was then filtered (Minisart SRP 15, PTFE-membrane, 0.45 µm) and concentrated (Amicon® Ultra-15, 10K cut off centrifugal filter device, Millipore) with distilled water (3 × 15 ml) in order to exchange solvent, remove DTT and unreacted

phosphite ligand. The resulting concentrated solution (150 µl) was diluted with distilled water to 1 ml.

Activity test
The residual activity of the enzyme was conveniently determined by monitoring the hydrolysis of Z-Gly-ONp as test substrate. This test was usually performed at various moments during the modification reaction. A sample of the reaction mixture (50 µl) was first diluted in phosphate buffer (950 µl, 100 mM, pH 7). A sample of this diluted solution (90 µl) was added to a quartz cuvette containing phosphate buffer (890 µl, 100 mM, pH 7). Subsequently the substrate was added (20 µl, 2.5 mM, acetone) and its hydrolysis was followed by UV (404 nm).[2]

Complexation of 5 with $Rh(COD)_2BF_4$ (6) and complex purification
A solution of $Rh(COD)_2BF_4$ (8 mM, 8 eq.) in dioxane (100 µl) was added to the aqueous solution (1 ml) containing modified papain (**5**) (0.1 mM). The resulting yellow solution was gently stirred for 1 h under nitrogen atmosphere at 25 °C. The complex was purified by size exclusion chromatography using desalting columns packed with polyacrylamide Bio-Gel® P-6DG gel (Econo-Pac® 10DG, Biorad). The sample was eluted with phosphate buffer (pH 7, 25 mM). This step allowed to exchange of water with a buffered solution and to remove excess Rh(I) present free in solution.
It turned out to be essential to perform the complexation step in pure water instead of buffered solution, as buffered solutions seem to cause the precipitation of the metal precursor. At the same time, it also appeared to be essential to perform the hydrogenation experiments in buffered solution, since the hybrid catalyst was not stable under hydrogenation conditions when in pure water.

Hydrogenation of methyl 2-acetamidoacrylate
In a typical hydrogenation run, a glass vial was charged with degassed buffer solution (2 ml, phosphate buffer, pH 7, 25 mM) containing the artificial metalloenzyme complex **5** (50 µM) and methyl 2-acetamidoacrylate (20 mM). The glass vial was placed in an autoclave and after purging with N_2 (3 × 5 bar) the system was pressurized with hydrogen (12 bar) and the reaction mixture was stirred at room temperature for 16 h. The reaction was stopped by release of the H_2 pressure. The resulting mixture was extracted with EtOAc (3 × 5 ml) and the combined organic layers were dried on Na_2SO_4. Conversion was determined by ^{1}H-NMR on a sample of the organic solution. Enantiomeric excess was determined by capillary chiral GC, using the following conditions:
CP Chirasil-L-Val column (25 m × 0.25 mm × 0.25 µm)
Init. Temp.: 110 °C, 12.5 min, 10 °C / min to 160 °C. $T_{det/inlet}$ = 250 °C, split ratio 25:1
t_R = 4.09 min, t_S = 4.78 min, t_{SM} = 2.47 min.

ESI-MS measurements
Electrospray mass spectrometry (ESI-MS) was performed on an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada): a triple quadrupole mass spectrometer supplied with an atmospheric pressure ionization source and a TurboIonSpray interface. The spectra were scanned in the range between m/z 1000.0 and

2800.0. The samples were diluted with an aqueous solution of MeOH (85%) containing 0.01% HCOOH.
The sequence of papain reported by Cohen *et al.* was used as reference.[3] The mass associated with this sequence is 23428 Da. Since 6 cysteines in the sequence are involved in disulfides bonds, the average mass considered was 23422 Da.

1. The first analysis was performed on activated papain, that was treated and purified according to the modification protocol but in the absence of the phosphite ligand. The observed mass (23455 Da) was assigned to papain in which the free cysteine is oxidized to the corresponding sulphinic acid.[4]
2. In the next analysis, papain was treated with 3-hydroxy-phenacyl bromide, used as a test reagent for the modification protocol. Moreover, this adduct results also in case of hydrolysis of the phosphite.
3. For ESI-MS analysis it is generally necessary to use 0.1% of formic acid. During the experiments conducted on modified papain **5**, it turned out necessary to reduce the amount of formic acid to 0.01%, as its presence caused hydrolysis of the phosphite ligand **4**. This significantly reduced the presence of the hydrolysis adduct. It is not clear whether the residual hydrolysis adduct with a mass of 23557 Da is still caused by the analysis conditions or already present in the sample. In the ESI-MS spectrum only a peak corresponding to the addition of one phosphite ligand was observed, confirming mono alkylation of the enzyme.
4. Finally, modified papain **5** complex with $Rh(COD)_2BF_4$ (**6**) was also analyzed by ESI-MS. The spectrum showed the disappearance of the peak at 24207 Da and the appearance of a peak at higher mass corresponding to the addition of Rh(COD) and possibly a molecule of water. This seems to signify the presence of a stable adduct between modified **5** and one equivalent of the rhodium precursor.

1. Papain (SO_2H) oxidized: expected 23454 Da, found 23455 Da.
2. Papain modified with 3-hydroxy-phenacyl bromide: expected 23556 Da, found 23557 Da.
3. Modification of papain with the phosphite ligand (**5**): expected 24207 Da, found 24207 Da.
4. Complex (**6**): expected 24435 Da, found 24434 Da.

Digestion of modified papain 5 with trypsin[5]
For the in-gel digestion, the native and modified papain protein bands were electrophoresed in SDS-PAGE gel and then cut out of the gel. For native papain, the mass of the peptide Asn^{18}-Lys^{39} (2270 Da) would increase to 2384 Da because both Cys22 and Cys25 were reacted with iodoacetamide (+57 Da). For modified papain **5** the mass of the same peptide Asn^{18}-Lys^{39} should be 3114 Da., because Cys 22 was modified by iodoacetamide, and Cys 25 was modified with the ligand (787 Da). An ion at m/z 1192.72 was found from the spectrum of native papain, corresponding to the doubly protonated ion of the peptide Asn^{18}-Lys^{39} (2384 Da) of native papain. However, the peptide with the complete ligand (3114 Da) was not observed in the spectrum of modified papain **5**. Alternatively, an ion at m/z 1232.75 was found instead, corrisponding to the doubly protonated peptide Asn^{18}-Lys^{39} with the linker part of the ligand (2270+57+136=

2463 Da): showing that the ligand was hydrolyzed and lost the phosphite part during the procedure.
To determine which cysteine residue of peptide ^{18}NQGSCGSCWAFSAVVTIEGIIAK^{39} (2463 Da) was modified by the phosphite ligand, the peptide was fragmented and analyzed by electrospray/tandem mass spectrometry. The expected b-type fragments were calculated for the peptides modified without ligand or with the ligand at either Cys22 or Cys25 (Fig. 1A) and compared with the fragmentation spectra. The product ion scan of the precursor ion m/z 1192.75 of the unmodified peptide displayed most of the possible b fragments (Fig. 1, A and B). The product ion scan of the $(M+2H)^{2+}$ ion at m/z 1232.75 of the modified peptide showed a mass increase of 78 Da of the b fragments starting at b_8, compared with the unmodified peptide (Fig. 1, A and C). The result confirmed that the ligand was positioned on Cys25 and not at Cys22.

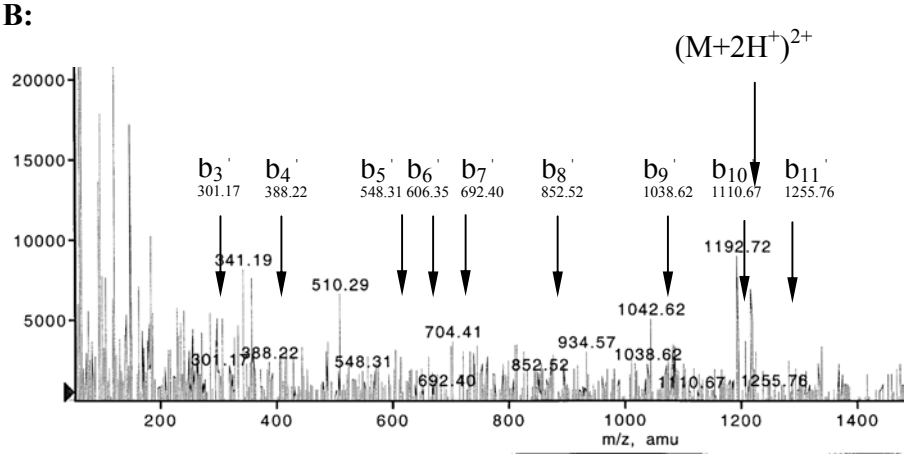
A:
Asn18 Gln19 Gly20 Ser21 Cys22 Gly23 Ser24 Cys25 Trp26 Ala27 Phe28
b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11}

Without ligand: (M = 2384 Da: Cys22 + 57 Da, Cys25 + 57 Da)
b ion masses 115.05 243.11 300.13 387.16 548.17 605.19 692.23 852.23 1038.31 1110.35 1255.42

With ligand:
If modified at **Cys22** (M = 2463 Da: Cys22 +136 Da, Cys25 +57 Da)
b ion masses 115.05 243.11 300.13 387.16 **625.17 683.19 770.23** 930.23 1115.31 1186.35 1333.42

If modified at **Cys25** (M = 2463 Da: Cys22 + 57 Da, Cys25 + 136 Da)
b ion masses 115.05 243.11 300.13 387.16 **548.17 605.19 692.23** 930.23 1115.31 1186.35 1333.42

B:



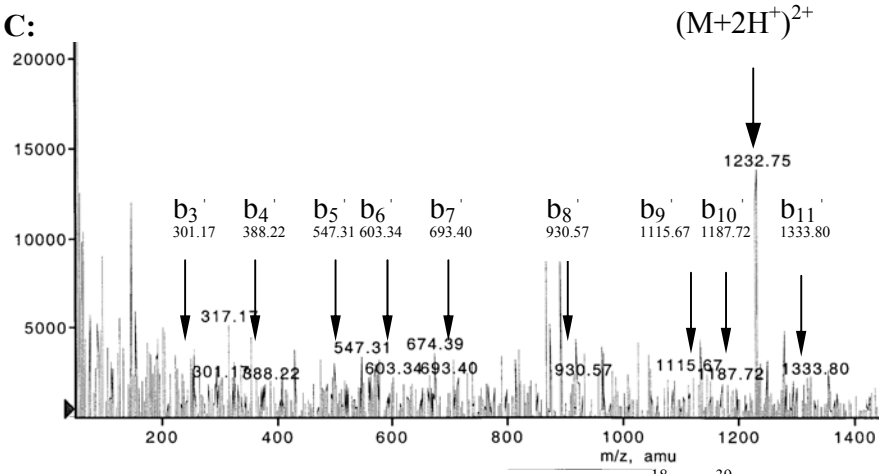


Fig. 1. Electrospray/tandem mass spectrometry of peptide Asn[18]-Lys[39]. **A**: peptide sequence and calculated monoisotopic singly charged masses for the b-type product ions of the unmodified and modified peptide at either position 22 or position 25. **B**: product ion spectrum of the precursor ion m/z 1192.72 from unlabeled papain. **C**: product ion spectrum of the precursor ion m/z 1232.75 from the labeled papain.

[1] A. Albeck and S. Kliper, *Biochem. J.*, 1997, **322**, 879.
[2] J. F. Kirsch and M. Ilgestrom, *Biochemistry*, 1966, **5**, 783.
[3] L. W. Cohen, V. M. Coghlan, and L. C. Dihel, *Gene*, 1986, **48**, 219.
[4] (a) G. Lowe, *Tetrahedron*, 1976, **32**, 291. (b) B. Biteau, J. Labarre, and M. B. Toledano, *Nature*, 2003, **425**, 980.
[5]In-gel digestion was performed essentially as previously described: A. Shevchenko, M. Wilm, O. Vorm, and M. Mann, *Anal. Chem.* 1996, **68**, 850.