

TECHNICAL NOTES BACHEM

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CHROMOPHORES/FLUOROPHORES: SPECTRAL PROPERTIES AND CHARACTERISTICS

Bachem offers series of peptide-based enzyme substrates linked to chromophores or fluorophores. The advantage of these chromogenic and fluorogenic substrates is the facile spectrometric detection and analysis of the reaction products. The following guide contains some useful information about the various kinds of substrates.

Chromophores

The characteristic feature of chromophores to absorb light of UV and visible wavelengths from 200 nm to 400 nm and from 400 nm to 800 nm, respectively, can be used to determine their concentration by absorption photometry. The method measures the decrease in light intensity when light passes through a colored solution. The distance which the light has to pass through a solution is called the path length. With a linearly rising concentration of the chromophore solution, the intensity of the emergent beam of light falls off exponentially.

The absorbance (A) is defined as follows:

$$A = \log I_0/I = \log 1/T$$

I	=	intensity of transmitted light
I_0	=	intensity of incident light
T	=	I/I_0 = transmittance

Since the absorbance (also called extinction or optical density (O.D.)) is linear to the concentration and the path length the Lambert-Beer's law can be applied (the law is limited since it is only valid for highly diluted solutions).

$$\text{Lambert-Beer's law} \quad A = \epsilon \times c \times d$$

A = absorbance	(dimensionless)
c = concentration	mol/l (M)
d = path length	cm
ϵ = molar decadic absorption coefficient	$l \text{ mol}^{-1} \text{ cm}^{-1} (\text{M}^{-1} \text{ cm}^{-1})^*$

*(the units for ϵ found in the literature are usually $\text{M}^{-1} \times \text{cm}^{-1}$)

Knowing the molar absorption coefficient of a chromophore in solution its concentration can be calculated according to the Lambert-Beer's law:

$$c = A/\epsilon \times d$$

Please note that molar absorption coefficients may depend on the temperature, pH, and the ionic strength of a solution.

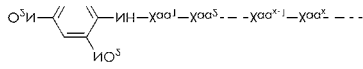
Table 1: Chromophores

Chromophore	Detection Wavelength*	Molar Absorption Coefficient*	References
Dnp (2,4-Dinitrophenyl)	365 nm	$\epsilon_{365\text{ nm}} = 17\,300\text{ M}^{-1}\text{cm}^{-1}$	[1] (for Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg OH (M-1340))
	354 nm	$\epsilon_{354\text{ nm}} = 16\,300\text{ M}^{-1}\text{cm}^{-1}$	[2] (for Dnp-Arg-Pro-Leu Ala-Leu-Trp-Arg-Ser-OH (M-2205))
	400 nm	$\epsilon_{400\text{ nm}} = 6\,100\text{ M}^{-1}\text{cm}^{-1}$	[3] (for Dnp (pH 4.2))
	400 nm	$\epsilon_{400\text{ nm}} = 6\,985\text{ M}^{-1}\text{cm}^{-1}$	[4] (for Dnp (pH 7.2))
FA (3-(2-Furyl)acryloyl) (decrease in absorbance measured)	322-345 nm	$\epsilon_{305\text{ nm}} = 24\,700\text{ M}^{-1}\text{cm}^{-1}$	[5] (for FA-Leu-Gly-Pro-Ala-NH ₂ (M-1385))
		$\Delta_{324\text{ nm}} = 2\,510\text{ M}^{-1}\text{cm}^{-1}$	[6] (for FA-Gly-Abu-NH ₂ (M-1835), FA-Gly-Val-NH ₂ (M-1820), and FA-Gly-Nva-NH ₂ (M-1830))
		$\epsilon_{322\text{ nm}} = 13\,400\text{ M}^{-1}\text{cm}^{-1}$ $\Delta_{322\text{ nm}} = 2\,300\text{ M}^{-1}\text{cm}^{-1}$	
βNA (β -Naphthylamide) (requires color reaction)	540 nm (detected by coupling with tetrazotized diorthoanisidine (Dialzo Blue B))		[7]
	560 nm (by the use of a modified Bratton-Marshall method)		
β-Naphthyl Ester (requires color reaction)	520 nm (detected by coupling to the zinc-stabilized diazonium salt, Fast Garnet)		[8]
pNA (p-Nitroanilide)	405 nm	$\epsilon_{405\text{ nm}} = 9\,450\text{ M}^{-1}\text{cm}^{-1}$	[9]
	410 nm	$\epsilon_{410\text{ nm}} = 8\,800\text{ M}^{-1}\text{cm}^{-1}$	[10]
ONp (p-Nitrophenyl ester)	347 nm	$\epsilon_{347\text{ nm}} = 5\,500\text{ M}^{-1}\text{cm}^{-1}$	[11]
SBzl (Thiobenzyl Ester) (requires color reaction)	324 nm (formation of 4-thiopyridone with 4,4'-dithiodipyridine)	$\epsilon_{324\text{ nm}} = 19\,800\text{ M}^{-1}\text{cm}^{-1}$	[12]
	410 nm (formation of 2-nitro-5-thiobenzoate anion with 5,5'-dithiobis-(2-nitrobenzoic acid))	$\epsilon_{410\text{ nm}} = 14\,000\text{ M}^{-1}\text{cm}^{-1}$	[13]

*the values listed are as reported in the cited literature.

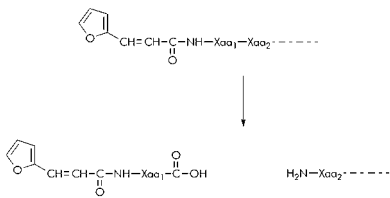
2,4-Dinitrophenyl (Dnp) Substrates

Dnp is normally used as a quencher for various fluorophores as indicated in Table 3. With some substrates (e.g. M-1340), however, enzymatic cleavage is assayed by measuring the absorption of the Dnp-linked peptide cleavage product at 365 nm after organic extraction from the assay buffer.



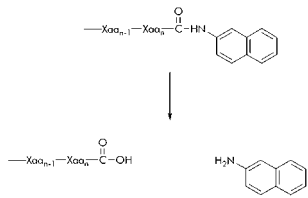
3-(2-Furyl)acryloyl (FA) Substrates

FA-linked substrates have been used for continuous spectrophotometric assays of various proteases such as collagenases, thermolysin, angiotensin I-converting enzyme, elastase, and carboxypeptidase Y. Assays with FA-peptide substrates are based on the blue shift in the near-ultraviolet absorption band of the FA-peptide when the peptide bond between the first and second residue is hydrolyzed. The resulting decrease in absorbance can be measured at wavelengths between 322 nm and 345 nm.



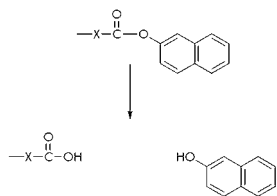
β -Naphthylamide (β NA) Substrates

β NA substrates can either be used as chromogenic or fluorogenic substrates. Photometry, however, requires coupling of the released β -naphthylamine to an azo dye (please see also 'Fluorophores').



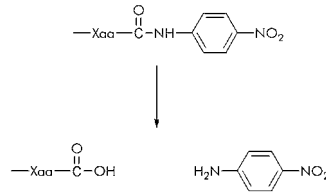
β -Naphthyl Ester Substrates

Hydrolytic activity using β -naphthyl ester substrates can be measured at 520 nm by coupling the liberated naphthol to the zinc-stabilized diazonium salt, Fast Garnet.



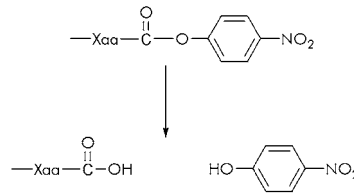
p-Nitroanilide (pNA) Substrates

pNA substrates are commonly used for the measurement of serine and cysteine proteases. The released p-nitroaniline shows a different absorption spectrum than pNA which is linked via an amide bond to the carboxy function of the peptide. p-Nitroaniline is either measured at 405 nm or 410 nm.



p-Nitrophenyl Ester (ONp) Substrates

In few substrates p-nitrophenyl is linked via an ester bond to the carboxyl group of an amino acid. Esterase activity can be measured spectrophotometrically at 347 nm. At this wavelength the absorption of p-nitrophenol ($\epsilon_{347\text{ nm}} = 5\,500\text{ M}^{-1}\text{ cm}^{-1}$) is independent of the pH.



Thiobenzyl Ester (SBzl) Substrates

Many commercially available substrates contain thiobenzylester groups which upon cleavage can be converted into a chromophore by using 5,5'-dithiobis-(2-nitrobenzoic acid) also known as DTNB or Ellman's reagent. The rate of thioester hydrolysis is measured at 405 nm to monitor the formation of 2-nitro-5-thiobenzoate anion ($\epsilon_{410\text{ nm}} = 14\,000\text{ M}^{-1}\text{ cm}^{-1}$). Alternatively, 4,4'-dithiodipyridine can be used to measure the thiolation degree by detecting the formation of 4-thiopyridone at 324 nm ($\epsilon_{324\text{ nm}} = 19\,800\text{ M}^{-1}\text{ cm}^{-1}$).

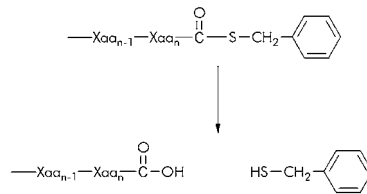


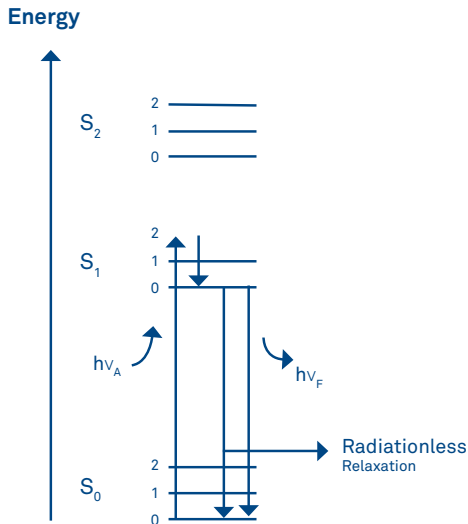
Table 2: Fluorophores

Fluorophore	Excitation Wavelength*	Emission Wavelength*	References
Abz (2-Aminobenzoyl or Anthraniloyl)	320 nm	420 nm	[14] [15] [16]
N-Me-Abz (N-Methyl-anthraniloyl)	340 - 360 nm	440 - 450 nm	[17]
AFC (7-Amido-4-trifluoromethylcoumarin)	395 nm 400 nm	495 nm 505 nm	[18] [19]
AMC (7-Amido-4-methylcoumarin)	360 nm 360 nm 380 nm 380 nm	440 nm 460 nm 440 nm 460 nm	[20] [21] [22] [10]
EDANS (5-[(2-Aminoethyl)-amino]naphthalene-1-sulfonyl)	340 nm	490 nm	[23]
FITC (Fluorescein isothiocyanate)	490 nm	520 nm	[24]
4MβNA (4-Methoxy-β-naphthylamide)	335 nm 340 nm 340 nm 350 nm	410 nm 425 nm 430 nm 440 nm	[25] [26] [27] [28]
Mca (7-Methoxycoumarin-4-yl)acetyl)	325 nm	392 nm	[29]
βNA (β-Naphthylamide)	320 nm 340 nm	420 nm 410 nm	[30] [31]
Trp (Tryptophan)	280 nm	360 nm	[14]

*the values listed are as reported in the cited literature.

Fluorescence

Fluorophores are substances which, like chromophores, absorb light in the UV or visible range. In contrast to chromophores they re-emit part of the light as radiation. This process is called fluorescence and can be illustrated by the energy level diagram suggested by A. Jablonski.



Absorption of light ($h\nu_A$) causes an electron to be promoted from its electronic ground state (designated as S_0) to an excited state (usually S_1). Every energy state has several vibrational energy levels 0, 1, 2 etc. During the lifetime of the excited state, i.e. the time elapsed between excitation of the molecule and emission of the photon (usually between 1-10 ns) part of the energy is lost by internal vibration. As a result the wavelength of the emitted light ($h\nu_F$) is always longer than that of the exciting light. This phenomenon is called the Stokes shift and allows the detection of emission against a background of light derived from excitation. Usually, the fluorescence excitation spectrum of a fluorophore in a diluted solution is identical to its absorption spectrum and under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength.

In a diluted solution, fluorescence intensity is linearly proportional to several parameters as deduced from the Lambert-Beer's law. These are the molar absorption coefficient, the path length, the intensity of the incident light, and the quantum yield which is the ratio of the number of emitted to the total number of absorbed photons.

Fluorescence detection is dependent on the sensitivity of the instrument and is therefore measured in arbitrary units.

Higher concentrations of the fluorophore (> 0.1 absorption units) lead to deviations from linearity due to loss of excitation intensity across the cuvette path length as the excitation light is absorbed

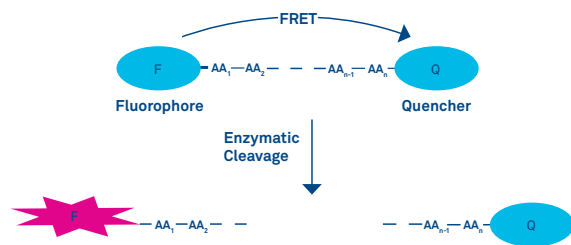
by the fluorophore. This phenomenon is known as the inner filter effect. Other effects which influence fluorescence measurements are related to intrinsic or background fluorescence originating from sample preparations and buffer contaminants, respectively. To minimize fluorescence derived from contaminants it is recommended to use materials of the highest purity.

Fluorescence spectra may also be dependent on the solvent. With some fluorophores such as 2-acetylthracene or tryptophan a spectral shift to longer wavelengths (bathochromic shift or red shift) is observed in more polar solvents. As mentioned with AMC (please see below) the pH of a solution may also change the fluorescence properties of a fluorophore.

Fluorescence Quenching

Any process which decreases the fluorescence intensity of a given substance can be referred to as quenching. Several types of quenching processes can be distinguished. These include collisional and static quenching, as well as fluorescence resonance energy transfer (FRET). Collisional or dynamic quenching can be considered as a reduction in fluorescence intensity due to a collision of the quencher with the fluorophore in the excited state. Upon contact the fluorophore returns to the ground state without light emission. One of the best known collisional quenchers which quenches almost all known fluorophores is molecular oxygen. It is therefore often required to remove dissolved oxygen to obtain reliable measurements. In static quenching a non-fluorescent complex is formed between the quencher and the fluorophore. In contrast to both of these quenching processes, FRET does not require contact of the quencher with the fluorophore. The energy transfer occurs without the appearance of a photon.

Fluorescence Resonance Energy Transfer (FRET)

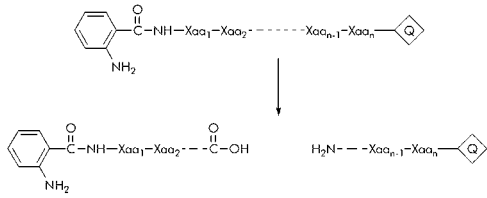


Fluorescence Resonance Energy Transfer (FRET)

Fluorescence energy transfer (FRET) is the transfer of the excited state energy of a donor to an acceptor without the emission of light. The energy transfer can be considered as an energy exchange of an oscillating dipole to a dipole with similar resonance frequency. In order to occur the emission spectrum of the donor has to overlap with the absorption spectrum of the acceptor, and the donor and acceptor have to be within a distance of 1-10 nm. The energy transfer efficiency depends on the extent of the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles, and the distance r between donor and acceptor. The energy transfer efficiency decreases exponentially by r^6 . The distance at which the efficiency of energy transfer is reduced by 50% is a characteristic value for a given donor acceptor pair and is called the Förster distance R_0 .

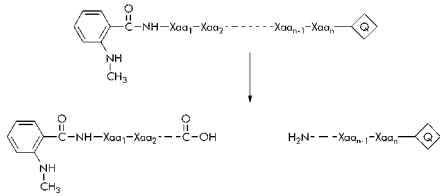
2-Aminobenzoyl or Anthraniloyl (Abz) Substrates

The Abz fluorophore is generally used in combination with a number of quenchers (Q) such as Dnp (2,4-dinitrophenyl), EDDnp (N-(2,4-dinitrophenyl)ethylenediamine), p-nitrophenylalanine, or 3-nitrotyrosine. Substrate cleavage can be detected at 420 nm using an excitation wavelength of 320 nm.



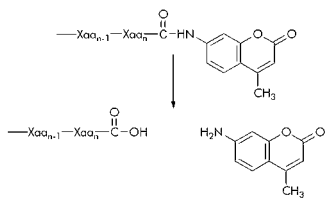
N-Methyl-anthraniloyl (N-Me-Abz) Substrates

N-Me-Abz substrates are generally used with Dnp as quencher (Q). The fluorescent group is either linked to the amino-terminal amino group or the ϵ -amino group of a lysine residue. Substrate cleavage can be detected at 440-450 nm using an excitation wavelength of 340-360 nm.



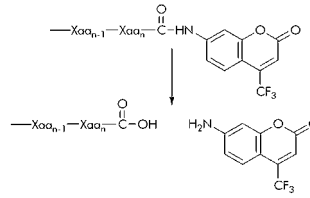
7-Amido-4-methylcoumarin (AMC) Substrates

Substrates with AMC attached to the C-terminal carboxyl group are commonly used for assaying carboxypeptidases. Cleavage of the amide bond between the peptide substrate and the AMC group results in the release of 7-amino-4-methylcoumarin which shows different excitation and emission spectra than the amide bond linked fluorogen. It can be detected at 440-460 nm using an excitation wavelength of 360-380 nm. Like other aromatic amines AMC is partially protonated at low pH (< 5) but fully deprotonated at physiological pH. Therefore, at or near physiological pH its fluorescence spectrum is pH-independent.



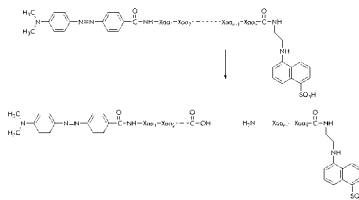
7-Amido-4-trifluoromethylcoumarin (AFC) Substrates

AFC substrates have the same basic structure as AMC substrates, the methyl group is replaced by a trifluoromethyl (CF_3) group. The release of 7-amino-4-trifluoromethylcoumarin is usually detected at 495-505 nm using an excitation wavelength of 395-400 nm. AFC substrates exhibit similar sensitivity as AMC substrates.



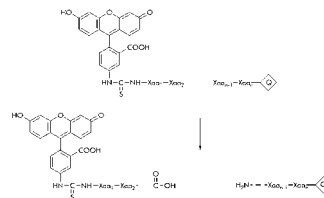
5-[(2-Aminoethyl)amino]naphthalene-1-sulfonyl (EDANS) Substrates

In these substrates, the fluorescence of the EDANS group is generally quenched by the DABCYL (4-(4-dimethylaminophenylazo)benzoyl) group. The DABCYL group is usually conjugated to the N-terminus and the EDANS group ($\epsilon_{305\text{nm}} = 24\,700\text{ M}^{-1}\text{cm}^{-1}$) attached to the C-terminus of the peptide substrate. Substrate cleavage can be detected at 490 nm using an excitation wavelength of 340 nm.



Fluorescein Isothiocyanate (FITC) Substrates

Only few FITC substrates have been described. The FITC label can be quenched with Dnp. Substrate cleavage can be detected at 520 nm using an excitation wavelength of 490 nm.



Below you can find a list of common donor acceptor pairs used for the design of enzyme substrates.

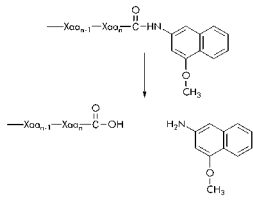
Table 3: Common Donor/Acceptor Pairs used at Bachem*

Donor	Acceptor (Quencher)	References
Abz (2-Aminobenzoyl or Anthraniloyl)	Dnp (2,4-Dinitrophenyl)	[14]
Abz (2-Aminobenzoyl or Anthraniloyl)	EDDnp (N-(2,4-Dinitrophenyl)ethylenediamine)	[32]
Abz (2-Aminobenzoyl or Anthraniloyl)	p-Nitro-phenylalanine	[33]
Abz (2-Aminobenzoyl or Anthraniloyl)	3-Nitro-tyrosine	[34]
N-Me-Abz (N-Methyl-anthraniloyl)	Dnp (2,4-Dinitrophenyl)	[17]
EDANS (5-[(2-Aminoethyl)amino]- naphthalene-1-sulfonyl)	DABCYL (4-(4-Dimethylaminophenylazo)benzoyl)	[23]
FITC (Fluorescein isothiocyanate)	Dnp (2,4-Dinitrophenyl)	[35]
Mca ((7-Methoxycoumarin-4-yl)acetyl)	Dnp (2,4-Dinitrophenyl)	[29]
Trp (Tryptophan)	Dnp (2,4-Dinitrophenyl)	[14]

*the values listed are as reported in the cited literature.

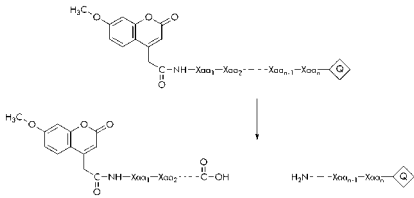
4-Methoxy- β -naphthylamide (4M β NA) Substrates

4M β NA substrates are commonly used for assaying protease activity. Cleavage of the amide bond between the peptide and 4M β NA results in the release of the fluorescent 4-methoxy- β -naphthylamine which can be detected at 410-440 nm using an excitation wavelength of 335-350 nm.



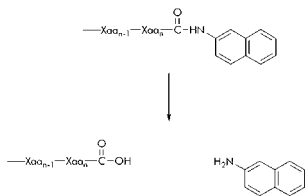
(7-Methoxycoumarin-4-yl)acetyl (Mca) Substrates

In this kind of substrates Mca is bound to an amino group (usually the N-terminal amino group) of a peptide sequence and quenched by Dnp (Q). The cleaved peptide fragment with the attached Mca group can be detected fluorometrically at 392 nm using an excitation wavelength of 325 nm.



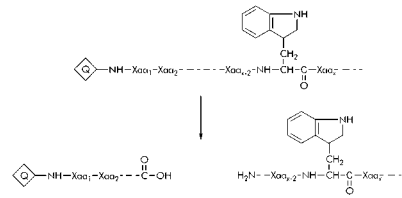
β -Naphthylamide (β NA) Substrates

As stated above (please see 'Chromophores') β NA substrates can either be used as chromogenic or fluorogenic substrates. The release of β -naphthylamine can be detected at 410-420 nm using an excitation wavelength of 320-340 nm.



Tryptophan (Trp) Substrates

Tryptophan (like tyrosine and phenylalanine) is a fluorescent amino acid which has been used in a variety of substrates with Dnp as a quencher (Q). Substrate cleavage can be detected at 360 nm using an excitation wavelength of 280 nm.



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