# Direct Carbodiimide-Mediated Conjugation of Carboxylates Using Pyridinium *p*-Toluenesulfonate and Tertiary Amines as Additives

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**Abstract:** The use of carboxylates in the carbodiimide-mediated coupling to amines was investigated. The addition of pyridinium *p*-toluenesulfonate (PPTS) and a tertiary amine was found to significantly improve acylation yields by up to 70%.

**Key words:** amides, coupling, carbodiimides, carboxylates, solid-phase synthesis

Carbodiimides are among the most popular activating reagents for carboxylic acids.[1] The formed active esters can react with nucleophiles leading to a large variety of possible products.[2] Despite the development of new coupling reagents,[3] e.g. of the aminium or phoshonium type, carbodiimide activation continues to be one of the most commonly used synthetic methods. Carbodiimides are in active use in the field of natural product synthesis[4] and offer interesting opportunities for chemoselective coupling reactions,[5] polymer grafting[6] or in reactions under the influence of microwaves.[7] In most cases amines are used as nucleophiles resulting in the formation of amides. Often carbodiimide activation serves as a practical means of accessing anhydrides and a variety of active esters.

For the described activation, the free acids are mandatory. Carboxylates, the salts of carboxylic acids, are unable to form the active esters in a completely aprotic environment. Nevertheless, there is interest in the direct activation of such salts. Many common chromophores are only commercially available as carboxylates or as preformed active esters, normally the hydroxysuccinimidyl esters. Usually, these active esters are by far more expensive than the corresponding carboxylates. For example, in our research efforts towards the development of biosensors we were in need of PNA-peptide conjugate **1** labeled with the DABCYL chromophore (Figure 1).

The introduction of DABCYL groups is commonly performed by using the commercially available *N*-hydroxysuccinimidyl ester **2**. This active ester is, however, 300-fold higher in price than the DABCYL sodium carboxylate **3** sold as methyl red (*para*). A direct use of carboxylates in coupling reactions would not only allow the use of inexpensive starting materials but would also offer advantages in natural product synthesis. Carboxylic acids are often protected as esters, which after a particular transformation has been achieved can be liberated by treatment with sodium- or potassium hydroxide solution. The conversion of the resulting sodium or potassium carboxylate to the free acid can be problematic due to acid labile protecting groups in other parts of the molecule. Apparently, the direct applicability of carboxylates in subsequent amidation or esterification reactions would be desirable.

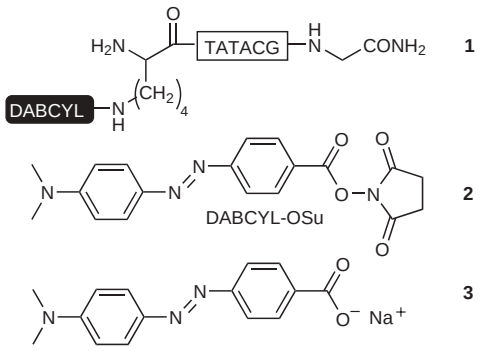
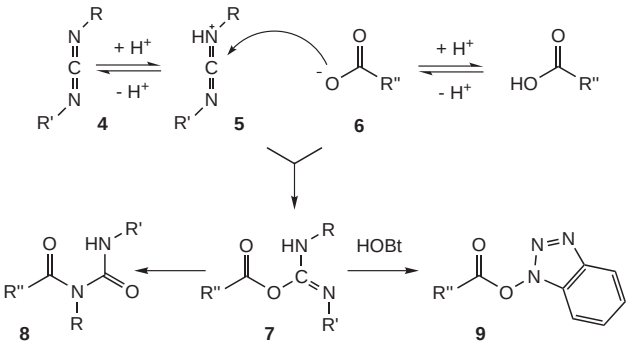



**Figure 1** DABCYL-labeled PNA-peptide conjugate and structures of the DABYL succinimidyl ester **2** and methyl red (*para*) **3**.

The carbodiimide-mediated activation of carboxylic acids is commenced by a protonation of the carbodiimide **4** (Scheme 1).[8] In a second step, the carboxylate **6** reacts with the protonated carbodiimide **5** to give an *O*-acylisourea **7**.[8] To avoid the rearrangement to *N*-acylurea **8**, usually HOBt is added, which by a transesterification finally leads to the desired active ester **9**.[9] Thus, the activation of carboxylic acid salts requires the presence of at least one equivalent of protons. However, addition of strong inorganic acids reduces the nucleophilicity of amines and carboxylate **6** and shall hence be avoided.




**Scheme 1** Carbodiimide-mediated activation of carboxylic acids.

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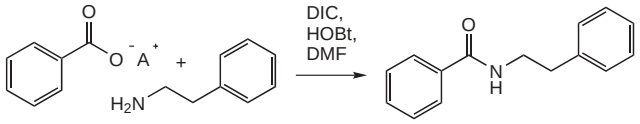
It was anticipated that the addition of the salt of a strong acid such as *p*-toluenesulfonic acid and a non-nucleophilic base like pyridine should in principle be able to deliver the necessary protons.[10] In the following we describe the development of efficient coupling reactions of carboxylic acid salts with amines that draw upon the surprisingly beneficial effect of PPTS/base mixtures.

The *N*,*N*′-diisopropylcarbodiimide (DIC)[11]/*N*-hydroxybenzotriazole (HOBt)-mediated coupling of potassium benzoate and benzoic acid with phenylethylamine was investigated as test reaction. Varying amounts of pyridinium *p*-toluenesulfonate (PPTS) and tertiary amine were added and product formation was analyzed by HPLC analysis of aliquots (Scheme 2).[12] Figure 2 shows coupling yields determined after four hours reaction time as a function of PPTS concentration.




**Scheme 2** Model system to test the influence of PPTS on the carbodiimide-mediated amidation (A = K, H).

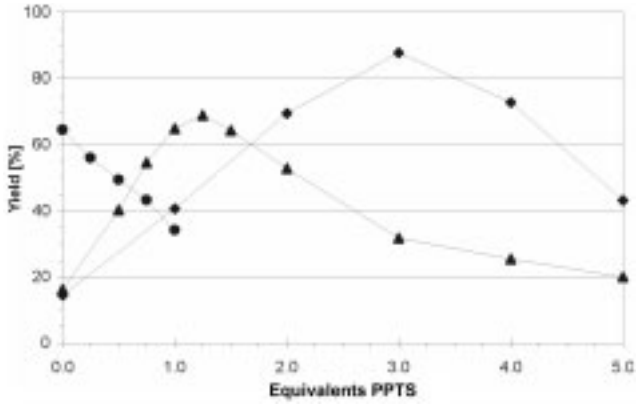



**Figure 2** Influence of PPTS. Reaction yields after four hours reaction time of the carbodiimide-mediated amide formation between benzoic acid (●), potassium benzoate (▲) or potassium benzoate with 2 equivalents of *N*-ethyldi*iso*propylamine (DIPEA) (♦) with phenylethylamine vs the amount of PPTS present. *Conditions*: 0.1 M potassium benzoate or benzoic acid, 0.1 M DIC, 0.1 M HOBt, 0.1 M phenylethylamine and the specified amount of PPTS in DMF.

Only minor amounts of benzoic acid phenethylamide (less than 20% yield) were formed in the absence of PPTS when using the carboxylate as coupling partner. The addition of 0.5 equivalents of PPTS led to an increase in yield by a factor of two. In the presence of 1.25 equivalents PPTS the yield was further increased reaching almost 70%. The addition of more than 1.25 equivalents of PPTS proved detrimental, presumably due to increasing protonation of the amine. In case benzoic acid was coupled, the addition of 1 equivalent of PPTS led to a two-fold decrease in yield, which confirmed the negative effect of overstoichiometric 'proton loads'. Next was studied the addition of PPTS in presence of a tertiary amine base. Interestingly, the addition two equivalents of di*iso*propylethylamine (DIPEA) resulted in an increase of the maximum reaction yield. The maximum yield was obtained with 3 equivalents of PPTS and amounted to almost 90% after four hours. At this stage, it is unclear why the PPTS/DIPEA mixture confers higher yields in the DIC/HOBt-mediated coupling of benzoate than PPTS alone. We note that the observed enhancements of coupling yields are independent of counterions (data not shown) and the carboxylate being used (vide infra).

It was next decided to investigate acyl donors used in labeling and staining chemistries. Figure 3 depicts useful dyes, all of which are characterized by low prices of the carboxylate [(eosin Y (**10**); erythrosine B (**11**) and phloxine B (**12**)]. Additionally, the coupling of two commonly used fluorescent dyes in the carboxylic acid form, FAM (**13**) and rhodamine B (**14**), was explored.

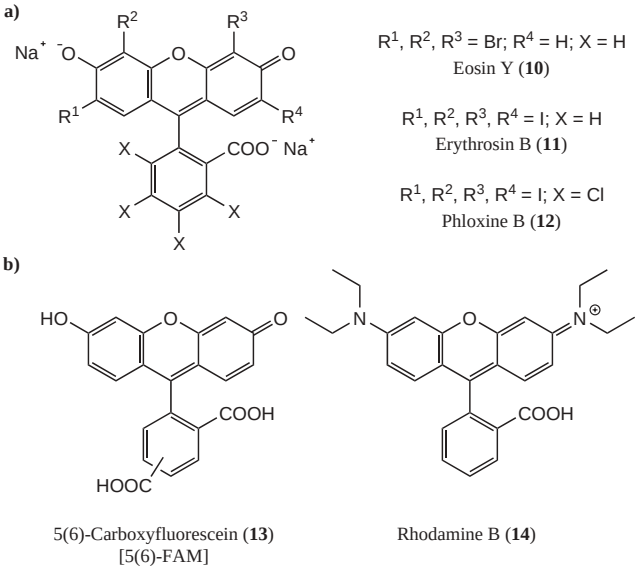



**Figure 3** Structures and names of the investigated dyes (**a**: dye carboxylic acid salts, **b**: dyes with free carboxylic acids).

Figure 4 illustrates the acylation yields revealed for the coupling of dyes **10**–**14** to phenylethylamine. Again, the presence of PPTS/DIPEA (3:2) resulted in pronounced improvements of coupling yields. For example, the yields of the coupling of the sodium salts eosin Y (**10**) and erythrosine B (**11**) were increased from 40% to 98% and 38% to 94%, respectively, upon addition of 3 equivalents of PPTS and 2 equivalents of DIPEA.

Remarkably, these improvements in coupling yields also were observed for acylation reactions with the free acids FAM (**13**) and rhodamine B (**14**). The coupling of **13** and **14** proceeded in 78% and 63% yield, respectively, while only 42% and 45% yield were determined for the reaction in the absence of PPTS/DIPEA. These results suggest a general advantageous effect of PPTS/DIPEA in carbodiimide-mediated amide bond formations. The described PPTS-effect is not limited to the carbodiimide DIC. Coupling reactions of potassium benzoate or rhodamine B

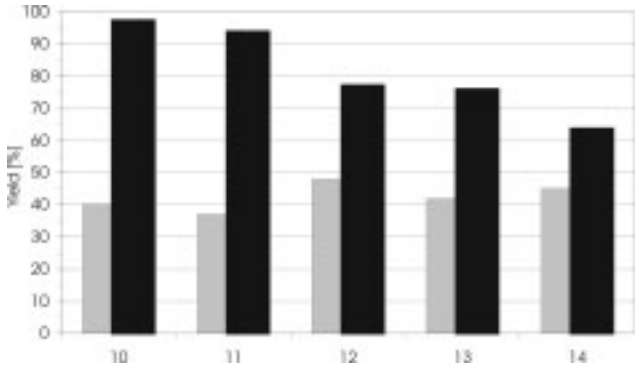
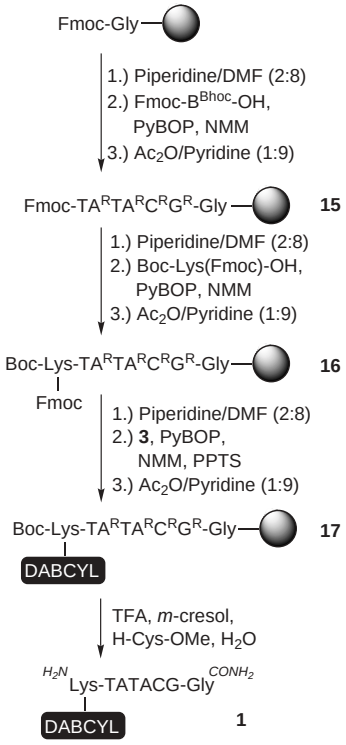



**Figure 4** Effect of PPTS/DIPEA on the reaction yield after four hours of the carbodiimide-mediated amide bond formation of common dyes with phenylethylamine in the absence (grey columns) and presence of PPTS/DIPEA (black columns). *Conditions*: 0.1 M dye, 0.1 M DIC, 0.1 M HOBt and 0.1 M phenylethylamine, and 0.2 M DIPEA and 0.3 M PPTS were added.

(**14**) with phenylethylamine promoted by the most popular *N*,*N*′-dicyclohexylcarbodiimide (DCC)[13] were similar (data not shown).

The optimized coupling conditions were applied in a synthesis of the DABCYL-labeled PNA-peptide conjugate **1** which was required for the application of a fluorescence resonance energy transfer (FRET)-based biosensor. It was furthermore desired to perform the entire synthesis on the solid phase in order to facilitate screening for alternative dyes (as FRET acceptors) if necessary.




**Scheme 3** Solid-phase synthesis of the labeled PNA-peptide conjugate by using a PPTS-aided coupling of DABCYL sodium salt **3** (R = benzhydryloxycarbonyl).

The solid-phase synthesis was carried out according to the Fmoc-strategy by using commercially available Fmoc/Bhoc-protected PNA monomers and Rink–Tentagel resin (Scheme 3). Treatments with piperidine in DMF removed the Fmoc-protecting groups. The PNA monomer building blocks were coupled by using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as activating agent in presence of *N*-methylmorpholine (NMM). Unreacted amino groups were blocked by acetylation. Boc/Fmoc-protected lysine was coupled to **15** as last building block. In preparing for the on-resin labeling the Fmoc-group on the lysine side-chain of resin **16** was removed. As penultimate step the conjugation with the DABCYL-group was performed. The DABCYL sodium salt **3** was allowed to react with the liberated side-chain amino group on the otherwise fully protected PNA-resin. As elaborated before, PPTS and a tertiary amine, in this case NMM, were added to the coupling cocktail. Finally, the DABCYL-PNA-peptide conjugate **1** was released by treatment of the resin **17** with TFA. After purification by preparative HPLC, conjugate **1** was furnished in 19% overall yield, which compares well to the 25–30% yield delivered by PNA routine synthesis. Moreover, the HPLC trace of the crude material obtained after cleavage from the resin and SepPak® filtration showed no unlabeled product (Figure 5). It can be concluded that the beneficial effect of mixtures of PPTS and a tertiary amine on acylation reactions is not limited to reactions in solution but can also be used for the direct conjugation of carboxylates to resin-bound nucleophiles.

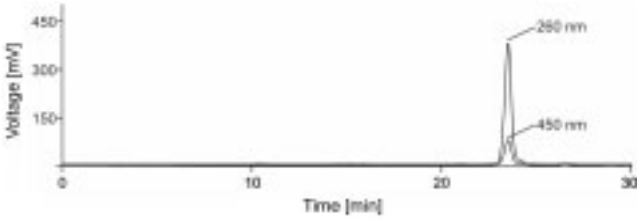



**Figure 5** HPLC-trace of the crude DABCYL-labeled PNA-peptide conjugate **1**.[14]

In concluding, the direct application of carboxylates in the most important carbodiimide-mediated reaction, the amide bond formation, was investigated. It was shown that the direct use of carboxylates is feasible and practical when a PPTS/base mixture is added to the amidation reaction. The addition of PPTS/base was found to significantly improve acylation yields, sometimes by up to 70%. This result is important since many useful chromophores are commercially available in inexpensive form as carboxylic acid salts. Nevertheless, even the carbodiimide-mediated coupling of free carboxylic acids was found to proceed with higher efficiency in presence of PPTS/base mixtures. The presented application of carboxylates for the on-resin labeling of biopolymers such as peptide nucleic acids offers new and inexpensive opportunities for

bioconjugation. Future studies will further investigate the applications of these findings for solid phase synthesis of peptides and peptide nucleic acids.

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(11) Schmidt, E.; Striewsky, W. *Chem. Ber.* **1941**, *74B*, 1285.
(12) (a) **General Procedure for the Carbodiimide-Mediated Amidation:** The carboxylic acid or carboxylate was diluted/suspended in DMF to yield a 0.1 M concentration. Under stirring HOBt (1 equiv), PPTS (specified amount), carbodiimide (1 equiv) and DIPEA (2 equiv when mentioned) was added. After 10 min 1 equiv of amine was added. The resulting mixture was stirred for 4 h and analyzed by RP-HPLC. (b) HPLC was performed on a Gilson 1105 HPLC-system (Nebula series) using a reversed phase Nucleodur C-18 Gravity 3 μm column (Macherey–Nagel, Düren) and a detection wavelength of 254 nm. Eluents: **A**: 98.9% $H_2O$, 1% MeCN (HPLC-grade, Biosolve BV, Valkenswaard, NL) 0.1% TFA (Peptide Synthesis Grade, Biosolve BV); **B**: 98.9% MeCN, 1% $H_2O$, 0.1% TFA. Gradient: 3–80% B in 20 min followed by 5 min 80% B (Phloxine B, Erythrosin B: Gradient: 3–100% B in 20 min followed by 5 min 80% B).
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(14) HPLC was performed on a Gilson 1105 HPLC-system (Nebula series) using a reverse-phase Nucleodur C-18 Gravity 3 μm column (Macherey–Nagel, Düren) and detection wavelengths of 260 nm and 450 nm. Eluents: **A**: 98.9% $H_2O$, 1% MeCN (HPLC-grade, Biosolve BV, Valkenswaard, NL) 0.1% TFA (Peptide Synthesis Grade, Biosolve BV); **B**: 98.9% MeCN, 1% $H_2O$, 0.1% TFA. Gradient: 3–30% B in 30 min.